Competing Application Example # 2

Note: This is a copy of a funded grant application. Permission from the Principal Investigator was obtained by NIH staff.

[redacted]

Form Approved Through 05/2004

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[redacted] of Health and Human Services
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ation

Do not exceed 56-character length restrictions, including spaces.

PI: [redacted]

1 R01 [redacted]

Dual: AG,ES,EY,MH,NS

IRG: ZRG1 ICP(02)

Council: 10/2002

Received: 04/22/2002

1 TITLE OF PROJECT

Tumor progression and apoptosis in mouse mammary gland

2 RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION ☐ NO ☒ YES
(If "Yes," state number and title)
Number: TW-02-002 Title: Global Health Initiative Program for New Foreign Investigators

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR | **New Investigator** ☐ No ☒ Yes

3a. NAME *(Last, first, middle)*
[redacted]

3b. DEGREE(S)
Ph. D.

3c. POSITION TITLE
Associate Researcher

3d. MAILING ADDRESS *(Street, city, state, zip code)*
[redacted]
(1425) Buenos Aires
Argentina

3e. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT
División Medicina Experimental, IIHEMA

3f. MAJOR SUBDIVISION
Academia Nacional de Medicina de Buenos Aires

3g. TELEPHONE AND FAX *(Area code, number and extension)*
TEL: 5411-4805-3411/5759 FAX: 5411-4803-9475

E-MAIL ADDRESS: [redacted]

4. HUMAN SUBJECTS RESEARCH
☒ No
☐ Yes

4a. Research Exempt ☒ No ☐ Yes
If "Yes," Exemption No.

4b. Human Subjects Assurance No.

4c. NIH-defined Phase III Clinical Trial ☒ No ☐ Yes

5. VERTEBRATE ANIMALS ☐ No ☒ Yes

5a. If "Yes," IACUC approval Date
NA in Argentina

5b. Animal welfare assurance no
None

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year—MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)
10/1/2002	10/1/2007	$50,000	$50,000	$250,000	$250,000

9. APPLICANT ORGANIZATION
Name [redacted]
Address : J. A. Pacheco de Melo 3081
(1425) Buenos Aires
Argentina

Institutional Profile File Number (if known)

10. TYPE OF ORGANIZATION
Public: → ☐ Federal ☐ State ☐ Local
Private: → ☒ Private Nonprofit
For-profit: → ☐ General ☐ Small Business
☐ Woman-owned ☐ Socially and Economically Disadvantaged

11. ENTITY IDENTIFICATION NUMBER
NA in Argentina
DUNS NO. *(if available)*
Congressional District

12. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
Name Christiane Dosne Pasqualini
Title Scientific Director, IIHEMA
Address : J. A. Pacheco de Melo 3081
(1425) Buenos Aires
Argentina

Tel :5411-4805-3411/5759 FAX 5411-4803-9475
E-Mail chdosne@hotmail.com

13. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name Christiane Dosne Pasqualini
Title Scientific Director, IIHEMA
Address : J. A. Pacheco de Melo 3081
(1425) Buenos Aires
Argentina

Tel :5411-4805-3411/5759 FAX 5411-4803-9475
E-Mail chdosne@hotmail.com

14. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PI/PD NAMED IN 3a *(In ink. "Per" signature not acceptable.)*
[redacted]

DATE
4/19/02

15. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 13. *(In ink. "Per" signature not acceptable.)*
[redacted]

DATE
4/19/02

[redacted]

DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. **DO NOT EXCEED THE SPACE PROVIDED.**

The present project addresses two main goals: 1) Discovering new pathways involved in mammary tumor progression, particularly those related to the loss of hormone-dependency; and 2) determine the events that initiate the cascades that trigger programmed mammary cell death during mammary gland involution. Understanding what determines the neoplastic-cell lack of response to the regulatory controls for cell proliferation and death is the main goal for experimental oncology. In the case of mammary cells, one of the main controls for proliferation and differentiation resides in the action of pregnancy-related hormones. Determine new genes and pathways that release the mammary epithelial cells from such a control is a fundamental issue in the fight against breast cancer. A particular aspect of this process will be focused in our studies: the fast and aggressive behavior of tumors that resume growth after long periods of dormancy. Although a relevant issue in the treatment of cancer patients, there has not been too many cellular or molecular approaches to this issue. Our studies will be carried out using new MMTV variants that induce pregnancy-dependent tumors that progress to a hormone-independent behavior. Using the Inverse PCR technique, the MMTV sequences will provide us a molecular tag for cloning host genomic regions that, when altered, contribute to tumor progression. It has been proposed that stimuli that trigger apoptosis in normal cells, would fail in neoplastic tissue. In the mammary gland, the process by which the lactating gland goes back to a virgin-like state is known as mammary involution. This process takes place after each lactation period and involves a very important reduction, by apoptosis, of the mammary alveolar epithelium. The signaling pathways that become activated in the mammary secretory cells right after weaning have received a lot of attention in the scientific community during the last years. However, the very early causes that determine the initiation of this process remain unknown. The purpose of the experiments described in our project is to study these early events to determine how the lack of suckling induces mammary cell death. This issue will be approached by in vivo as well as in vitro experiments. In addition, we will focus in determining whether neoplastic cells show alteration in the signaling pathways that lead to mammary epithelium cell death and whether that would be relevant during tumor progression.

PERFORMANCE SITE(S) *(organization, city, state)*
División Medicina Experimental, Instituto de Investigaciones Hematológicas (IIHEMA), Academia Nacional de Medicina Buenos Aires, Argentina.

[redacted]

KEY PERSONNEL. See instructions. *Use continuation pages as needed* to provide the required information in the format shown below. Start with Principal Investigator. List all other key personnel in alphabetical order, last name first.

Name	Organization	Role on Project
[redacted]	IIHEMA, Academia Nacional de Medicina	PI
Clrio, María Cecilia	IIHEMA, Academia Nacional de Medicina	Research assistant
Gattelli, Albana	IIHEMA, Academia Nacional de Medicina	Post-graduate fellow
Quaglino, Ana	IIHEMA, Academia Nacional de Medicina	Post-graduate fellow
Schere-Levy, Carolina	IIHEMA, Academia Nacional de Medicina	Post-graduate fellow

Disclosure Permission Statement. Applicable to SBIR/STTR Only. See instructions. ☐ Yes ☐ No

[redacted]

RESEARCH GRANT
TABLE OF CONTENTS

BUDGET JUSTIFICATION PAGE MODULAR RESEARCH GRANT APPLICATION				
Initial Budget Period	Second Year of Support	Third Year of Support	Fourth Year of Support	Fifth Year of Support
$ 50,000	$ 50,000	$ 50,000	$ 50,000	$ 50,000
Total Direct Costs Requested for Entire Project Period				$ 250,000

Personnel

Edith Kordon, Ph. D. (90% effort) will oversee the experiments contained within this proposal. In addition, Dr. Kordon will be the main responsible for surgical procedures.

Carolina Schere- Levy, Post-graduate fellow/Ph. D. student (100% effort). Lic. Schere-Levy will determine the activation of different signaling pathaways during mammary gland involution. She will also investigate which of these pathways could be triggered by LIF LIF (Leukemia inhibitory factor) in the pellet implanted mammary glands. She will also determine LIF activity on mammary epithelial cell lines . In order to carry out these studies she will performed the required molecular and immunohistochemical analysis.

Ana Quaglino, Post-graduate fellow/Ph. D. student (100% effort). She will test the effect of stretching on mammary epithelial cell cultures (cell lines and/or primary cultures). Besides, she will perform the experiments in transfected cells to study LIF expression regulation. She will also determine the effect of LIF on mammary tumors *in vivo* and *in culture* . In addition she will be in charge of testing the treatment with antisense-nucleotides *in vivo*.

Albana Gattelli, Post-graduate fellow, Ph. D. student (100% effort), is in charge of cloning the viral insertion sites in the MMTV-induced mammary tumors. She will also test the mutation and expression of the mutated loci in the tumor samples. She will also perform the molecular analysis of tumors that resumed growth after dormancy.

Maria Cecilia Cirio, Assitant researcher/Pregraduate student (30% effort). She will be in charge of following the animals implanted with mammary tumors. She will also collaborate in the immunohistochemical assays. When she graduates, in 2003, her effort will increase to 80-100% and she will collaborate in the analysis of mutated regions found by Gattelli.

To be Appointed Pathologist (20% effort), will collaborate with the histological (morphological and immunohistochemical) analysis of neoplastic and normal mammary samples.

Consortium

No consortium/contractual arrengment have been made up to the moment with any individual or organization. However, the economical situation in Argentina indicates that certain services and/or technical assistance provided by our Institute would have to be paid by the researcher's grants in the coming future.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
[redacted]	Associate Researcher

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Buenos Aires, Argentina	MS in Biology	1987	Animal Physiology
University of Buenos Aires, Argentina	Ph. D.	1991	Experimental oncology
National Cancer Institute, NIH, Bathesda, MD, USA		1992-1997	Mammary gland biology

A. Positions and Honors:

Positions and Employments:

1986-1987 Pregraduate research assistant at the National Academy of Medicine, Buenos Aires, Argentina. Supervisors: Claudia Lanari, Ph. D. Christiane Dosne Pasqualini, Ph. D.

2001-2002 Post-graduate research Fellow supported by the National Council of Scientific and Technological Research (CONICET), Argentina. Supervisors: Claudia Lanari, Ph. D. Christiane Dosne Pasqualini, Ph. D.

1992-1997 Post-doctoral Fogarty Fellow at the Laboratory of Tumor Immunology and Biology, Natl. Cancer Institue. National Institutes of Health, Bethesda, Maryland, USA. Supervisors: Robert Callahan, Ph. D. and Gilbert H. Smith, Ph. D.

1998-present Member of the National Council of Scientific and Technological Research (CONICET), Investigator Career. Position: Associate Researcher.

Other Experience and Professional Memberships

2001-2002 Member of the American Society of Cell Biology

1998-present Member of the Argentine Society of Clinical Investigation (SAIC).

1999-present Reviewer for fellowships, grants and career membership evaluation for the National Council of Scientific and Technological Research (CONICET), Argentina.

2000-present Member of the Bio-safety Committee at the Institute of Hemathological Research, National Academy of Medicine of Buenos Aires, Argentina.

2000-2001 Member of the Medical Sciences Study Section for fellowship evaluation for the National Council of Scientific and Technological Research (CONICET), Argentina.

2001 Member of the Comitee for fellowship evaluation for Antorchas Foundation, Argentina.

2002 International Union Against Cancer (UICC) fellow for the International Technical Exchange Program. University of Massachusetts Medical School, Worcester. MA, USA. February-March, 2002. Supervisor: Lucio H. Castilla, Ph. D.

Honors

1989 Premio "Centenario de la Colectividad Judía en la Argentina" ("A century of Jewish congregation in Argentina" Award for the best scientific work in Cancer: "Adenocarcinomas inducidos por acetato de medroxiprogesterona: un modelo murino" (Mammary adenocarcinomas induced medroxiprogestorone acetate: a murine model) Lanari C., **Kordon E.**, Elizalde P., Molinolo A. A., Charreau E. H. y Dosne Pasqualini C.

1995 Premio Accesit/ Lalcec (Angentine Ligue of Fight Against Cancer) "Mecanismos regulatorios en la carcinogénesis mamaria murina por progestágenos" (Regulatory mechanisms in mammary carcinogenesis by. Autores: Molinolo A., **Kordon E.**, Pazos P., Montechia M. F., Guerra F., Elizalde P., Luthy I., Charreau E. H., Dosne Pasqualini C. y Lanari C.

1996 ASCB (American Society for Cell Biology)/ Glenn Foundation Award "Evidence for a pluripotent mammary epithelial stem cell capable of self-renewal". Authors: Gilbert H. Smith and **Edith C. Kordon**. San Francisco, California, USA., December 8th.

Selected peer-reviewed publications (in chronological order)

Publications selected from a total of 18 peer-reviewed publications

Kordon E., Lanari C., Molinolo A.A., Elizalde P., Charreau E.H., Dosne Pasqualini C. D. Estrogen inhibition of MPA-induced mouse mammary tumor transplants. **Int. J. Cancer 49: 900-905, 1991**.

Jhappan C., Geiser A., **Kordon E.**, Bagheri D., Hennighausen L., Roberts A., Smith G. H., Merlino G. Targeting expression of Transforming Growth Factor β1 Transgene to the pregnant mammary gland inhibits alveolar development and lactation. **EMBO J. 12, 1835-1845., 1993**.

Kordon E., Guerra F., Molinolo A. A., Elizalde P., Charreau E., Dosne Pasqualini C. D., Montecchia F., Pazos P., Dran G., Lanari C. Effect of sialoadenectomy on medroxyprogesterone-acetate induced mammary carcinogenesis in BALB/c mice. Correlation between histology and epidermal- growth-factor receptor content. **Int. J. Cancer 59, 196-203, 1994.**

Kordon E., Mc Knight R., Jhappan C., Hennighausen L., Merlino G., Smith G. H. Ectopic TGFb1 expression in the secretory mammary epithelium induces early senescence of the epithelial stem cell population. **Dev. Biol. 168, 47-61, 1995.**

Kordon E., Smith G.H., Callahan R., Gallahan, D. A novel non-MMTV activation of the Int-3 gene in a spontaneous mouse mammary tumor. **J. Virol. 69, 8066-8069, 1995.**

Gallahan D.,Jhappan C., Robinson G.,Hennighausen L, Sharp R., **Kordon E.**, Callahan R., Merlino G., Smith G.H. Expression of a truncated Int3 gene in developing secretory mammary epithelium specifically retards lobular differentiation resulting in tumorigenesis. **Cancer Res. 56,1775- 1785, 1996.**

Kordon E., Smith G.H. An entire functional mammary gland may comprise the progeny from a single cell. **Development 125: 1921-1930, 1998.**

Buggiano V., Schere-Levy C., Abe K., Vanzulli S., Piazzon I., Smith G. H., and **Kordon E.** Impairment of mammary lobular development induced by expression of TGFβ1 under the control of WAP promoter does not suppress tumorigenesis in MMTV-infected transgenic mice. **Int. J. Cancer 92: 568-576, 2001.**

Rasmussen S., **Kordon E.**, Callahan R., Smith G. H. Evidence for the transforming activity of a truncated Int6 gene, in vitro. **Oncogene 20: 5291-5301, 2001.**

Buggiano V., Schere-Levi C., Gattelli A., Cirio MC, Marfil M, Nepommaschy I, Piazzon I, Helguero L, Vanzulli S. and **Kordon E.** Origin and Progression of Pregnancy-Dependent Mammary Tumors Induced by New Mouse Mammary Tumor Virus Variants. Breast Cancer Res. and Treat.. 2002. In press.

C. Research Support

Ongoing Research Support

2001-2003 Junior Investigator Award. Subject: "Pregnancy-dependent mouse mammary tumors induced by new MMTV variants. Study of origin and progression". **National Agency for Science and Technology Promotion, Argentina.** Role: PI.

2001-2002 Ramón Carrillo-Arturo Oñativia 2001, Junior Investigator Award. "Searching for new factors involved in mouse mamary gland normal and neoplastic development"**National Secretary of Public Health, Argentina.** Role: PI

2001-2002 Basic Research Award in Development. "LIF (Leukemia Inhibitory Factor) role during mammary gland development". **Antorchas Foundation, Argentina.** Role: PI.

2001-2003 Basic Research Grant Award. Subject: "Biology and tumorigenicity of Mouse Mammary Tumor Virus (MMTV) new variants". **National Agency for Science and Technology Promotion, Argentina.** Role: Co-investigator.

2001-2003 Medical Basic Research Award. Subject: "Role of protein Gα12 in mammary gland noral and neoplastic development". **Alberto J. Roemmers Foundation, Argentina.** Role: PI.

Completed Research support

1998-2000 Medical Basic Research Award. **Alberto J. Roemmers Foundation, Argentina.** Subject: "Effect of WAP-TGFβ1 expression on MMTV-induced mouse mammary tumorigenesis". Role: PI

2001-2001 Ramón Carrillo-Arturo Oñativia 2001. Junior Investigator Award. Subject: "Role of mammary stem cells in mammary gland neoplasia". **"National Secretary of Public Health, Argentina.** Role: PI.

1999-2000 Basic Research Award in Development. Subject: "LIF (Leukemia Inhibitory Factor) role during mammary gland development". **Antorchas Foundation, Argentina.** Role: PI.

1999 Re-entry Grant. Award for post-doctoral fellows that have returned to the country. Subject: New aspects of mammary gland development. **Antorchas Foundation, Argentina.** Role: PI.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Schere-Levy, Carolina P.	Post-graduate Fellow

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Buenos Aires (UBA)	M.Sc. Biology	1991-1997	Molecular Biology
University of Buenos Aires (UBA)	Ph. D Student 80% completed	1999-2003	Mammary gland biology

A. Positions and Honors.

1995 Pregraduate Research Assistant: Signal transduction laboratory. Institute of Biochemical Research Luis F. Leloir, Campomar Foundation. Signal transduction of Leukemia Inhibitory factor. Supervisor: Luis Jimenez de Asúa, MD, Ph.D

1995-1996 Selected to participate as a winter student at the Weizmann Institute of Science, Rehovot, Israel. Role of cytokines and growth factor in p53 induced apoptosis of granulosa cells. Supervisor: Professor Abraham Amsterdam.

1999-present National Scientific Research Council Post-graduate Fellow (CONICET). Department of Experimental Medicine. National Academy of Medicine from Buenos Aires. Supervisor: Edith C. Kordon, Ph.D

B. Selected peer-reviewed publications.

- Leukaemia Inhibitory Factor Induces Mitogenesis in Swiss 3T3 Cells and Selective Enhancement via a Variety of Signalling Events. Carolina Schere Levy, Moira Sauane, Philip S. Rudland and Luis Jimenez de Asua. 1997 *Biochemical and Biophysical Research Communication* 236, 814-818.

- Steroid and Aging. Abraham Amsterdam, Ada Dantes, Kumico Hosokawa, Carolina Schere Levy, Fumikazu Kotsuji and Dorit Aharoni. 1998 *Steroids* 63, 314-318.

- Modulation of Mdm2 Expression and p53-Induced Apoptosis in Immortalized Human Ovarian Granulosa Cells. Kumico Hosokawa, Dorit Aharoni, Ada Dantes, Eitan Shaulian, Carolina Schere

Levy, Ruth Atzmon, Fumikazu Kotsuji, Moshe Oren, Israel Vlodavsky and Abraham Amsterdam. 1998 *Endocrinology* vol.139, No 11, 4688-4700.

- Induction of Ad4BP/SF-1, Steroidogenic Acute Regulatory Protein and Cytochrome P450scc Enzyme System Expression in Newly Established Human Granulosa Cell Lines. Kumico Hosokawa, Ada Dantes, Carolina Schere Levy, Amihai Barash, Yoshio Yoshida, Fumikazu Kotsuji, Israel Vlodavsky and Abraham Amsterdam. 1998 *Endocrinology* vol.139, No 11, 4679-4687.

- Wap-TGF β1 mouse transgenic model shows that MMTV-oncogenic transformation occurs mainly in the undifferentiated mammary stem cell population. Valeria Buggiano, Carolina Schere Levy, Keiji Abe, Isabel Piazzon, Gilbert H. Smith and Edith Kordon. 2001 *International Journal of Cancer*, 92, 568-76.

- Origin and progression of pregnancy-dependent mammary tumors induced by new mouse mammary tumor virus variants. Valeria Buggiano*, Carolina Schere-Levy*, Albana Gattelli*, M. Cecilia Cirio, Mariana Marfil, Irene Nepomnaschy, Isabel Piazzon, Luisa Helguero, Silvia Vanzulli, Edith C. Kordon. 2002. Breast Cancer Res. Treat. In press. *These authors equally contributed to this paper.

Peer reviewed abstracts, presented in scientific meeting and published in international journals:

La muerte celular del epitelio secretorio mamario inducido por la expresión de TGFβ1 no afecta la actividad tumorigénica del virus de tumor de mama murino (MMTV). Carolina Schere-Levy, Valeria Buggiano y Edith Kordon. Medicina (Buenos Aires), vol.58 N° 5/2, 1998.

Estudios sobre origen celular y hormono-dependencia de tumores inducidos por nuevas variantes del virus de tumor mamario de ratón (MMTV). Carolina Schere-Levy, Valeria Buggiano y Edith Kordon. Medicina (Buenos Aires), vol.58 N° 5/2, 1998.

La infección de hembras transgénicas para WAP-TGFb1 con el virus del tumor mamario de ratón (MMTV) resulta en la inducción de tumores mamarios y la reversión del fenotipo senescente. Valeria Buggiano, Carolina Schere-Levy, Marcela Franco, Cecilia Cirio, Gilbert Smith, Edith Kordon. Medicina (Buenos Aires), vol.59 N° 5/2, 1999.

Inserciones del virus del tumor mamario murino (MMTV) como herramienta de estudio de la progresión tumoral. Albana Gatelli, Carolina Schere-Levy, Mariana Marfil, M. Cecilia Cirio, Valeria Buggiano y Edith Kordon. Medicina (Buenos Aires), vol. 60 N° 5/2, 2000.

Estudios sobre la participación del Factor Inhibidor de Leucemia (LIF) en el desarrollo de la glándula mamaria normal y neoplásica. Carolina Schere-Levy; Valeria Buggiano y Edith Kordon. Medicina (Buenos Aires), vol. 60 N° 5/2, 2000.

El Factor Inhibidor de Leucemia (LIF) participa en la involución de la glándula mamaria. Carolina Schere-Levy, Valeria Buggiano, Silvia Vanzulli y Edith Kordon. Medicina (Buenos Aires), vol. 61 N° 5/2, 2001.

C. Research Support.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Quaglino Ana	Graduate Fellow

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Buenos Aires	M.S. Biology	2001	Cell & Molecular Biology

A. Positions and Honors.

Positions and Employment

1994-1998 Computing & research assistant, Department of Interventional Cardiology, Buenos Aires Cardiovascular Institute, Buenos Aires, Argentina. Supervisor: Jorge Albertal, MD, Luis Guzmán, MD & Jorge Belardi, MD

1998-1999 Undergraduate research assistant. CIPYP (Porfiria and Porphirins Center Research).Facultad de Ciencias Exactas y Naturales (School of Sciences).. University of Buenos Aires, Argentina.
Supervisor: Ana Adela Juknat, Ph.D.

1999-2002 Undergraduate research assistant. Biological Chemistry Department. Facultad de Ciencias Exactas y Naturales (School of Sciences). University of Buenos Aires, Argentina.
Supervisor: Ana Adela Juknat, Ph.D & Mónica Kotler, Ph.D

2001 Teaching assistant. "Biochemistry and molecular biology Technics". Institute of Biochemical Research., Buenos Aires, Argentina.

2001 Teaching assistant. "Apoptosis and regulation of cell survival by neurotrophic factors in the nervous system". Biological Chemistry Department, Facultad de Cs. Exactas y Naturales (School of Sciences), University of Buenos Aires, Argentina

2002 Teaching assistant in Biological Chemistry. Facultad de Ciencias Exactas y Naturales, University of Buenos Aires, Argentina

2002-present Post-graduate research fellow. Supported by the National Secretary of Public Health (Argentina) at the National Academy of Medicine from Buenos Aires, Argentina. Supervisor: Edith Kordon, Ph. D.

B. Selected peer-reviewed publications

Peer reviewed abstracts, presented in scientific meeting and published in international journals.

"Caspases involved in apoptotic cell death induced by Hydrogen Peroxide in Astrocytes"
A Quaglino, MV Armanino, ML Kotler and AA Juknat. (2001) Cel. Mol. Neurobiol. Vol 21 (2), 158.

"Apoptotic Cell-death induced by Hydrogen Peroxide in Astrocytes. JNK activation".
MV Armanino, A Quaglino, AA Juknat and ML Kotler. (2001) Cel. Mol. Neurobiol. Vol 21 (2), 125.

"Hydrogen Peroxide - induced Apotosis involves the Processing of Caspases in Astrocytes".
A Quaglino, MV Armanino, ML Kotler and AA Juknat. (2001) J. Neurochem. 78: Supl. 1, 443

"Melatonin prevents Hydrogen Peroxide-induced apoptosis in Astrocytes. Implications of c-JUN N-terminal Kinase and Bax" M.V. Armanino, A Quaglino , A.A. Juknat and ML Kotler.
(2001) J. Neurochem. 78: Supl. 1, 443

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Gattelli, Albana	Graduate fellow

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
National University of Quilmes (UNQ), Pcia de Buenos Aires, Argentina.	M. S. Biotechnologist	2000	Biotech/Molecuar Biology

A. Positions and honors

2000-present Post-graduate research fellow: Supported by the National Secretary of Public health (Argentina) and Avon Foundation. Supervisor: Edith Kordon, Ph. D.

June-August 2001 "Exchange Visitor" University of Massachusetts Medical School (UMASS),Worcester, MA, United States.). Supervisor: Lucio Castilla, Ph. D.

November 2001 Teaching assistant: "Molecular Techniques for Identifying and Characterizing of viral receptors in Cos and 293T cells", National Academy of Medicine, Buenos Aries, Argentina. Invited professor: Susan Ross, Ph. D. University of Pennsylvania, USA).

B. Selected peer-reviewed publications

1.Buggiano V*, Schere-Levi C*, Gattelli A*, Cirio MC, Marfil M, Nepommaschy I, Piazzon I, Helguero L, Vanzulli S, Kordon E. Origin and Progression of Pregnancy-Dependent Mammary Tumors Induced by New Mouse Mammary Tumor Virus Variants. Breast Cancer Research and Treatment. 2002. In press.
*These authors equally contributed to this paper.

Peer reviewed abstracts, presented in scientific meeting and published in international journals.

1. Inserciones del virus del tumor mamario murino (MMTV) como herramienta de estudio de la progresión tumoral. Albana Gatelli, Carolina Schere-Levy, Mariana Marfil, M. Cecilia Cirio, Valeria Buggiano, Edith Kordon. Medicina (Buenos Aires), vol. 60 N° 5/2, 2000.

2. Caracterización histológica tumoral y clonado de loci afectados por el virus del tumor mamario murino (MMTV). Albana Gattelli, Valeria Buggiano, Cecilia Cirio, Luisa A. Helguero, Silvia Vanzulli, Lucio Castilla, Edith Kordon. Medicina (Buenos Aires), vol. 6 N° 5/2, 2001.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Cirio, Maria Cecilia	Pre-graduate student

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Buenos Aires	MS student	1998-present	Biology

A. Positions and employments:

1999-present Pregraduate research assistant at the National Academy of Medicine. Buenos Aires, Argentina. Supervisor: Edith C. Kordon, Ph. D.

B. Selected peer-reviewed publications

1.Buggiano V*, Schere-Levi C*, Gattelli A*, Cirio MC, Marfil M, Nepommaschy I, Piazzon I, Helguero L, Vanzulli S, Kordon E. Origin and Progression of Pregnancy-Dependent Mammary Tumors Induced by New Mouse Mammary Tumor Virus Variants. Breast Cancer Research and Treatment. 2002. In press.
*These authors equally contributed to this paper.

Peer reviewed abstracts, presented in scientific meeting and published in international journals.

La infección de hembras transgénicas para WAP-TGFβ1 con el virus del tumor mamario de ratón (MMTV) resulta en la inducción de tumores mamarios y la reversión del fenotipo senescente. Valeria Buggiano, Carolina Schere-Levy, Marcela Franco, Cecilia Cirio, Gilbert H. Smith, Edith Kordon. Medicina (Buenos Aires), vol.59 N° 5/2, 1999.

Inserciones del virus del tumor mamario murino (MMTV) como herramienta de estudio de la progresión tumoral. Albana Gatelli, Carolina Schere-Levy, Mariana Marfil, M. Cecilia Cirio, Valeria Buggiano y Edith Kordon. Medicina (Buenos Aires), vol. 60 N° 5/2, 2000.

Caracterización histológica tumoral y clonado de loci afectados por el virus del tumor mamario murino (MMTV). Albana Gattelli, Valeria Buggiano, Cecilia Cirio, Luisa A. Helguero, Silvia Vanzulli, Lucio Castilla, Edith Kordon. Medicina(Buenos Aires), vol. 6 N° 5/2, 2001.

RESOURCES

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory:
Our Division counts with four laboratories, two of them for mouse work, one for cell culture work and the fourth one for molecular analysis work. We share these laboratories with another three groups of approximately the same size of ours. These laboratories are fully available for our work

Clinical:
NA

Animal:
Our mouse colony counts with seven air conditioned rooms at 20 + 20C, under an automatic 12h light/12 hour darkness schedule. The mice are maintained in a pathogen-free environment. They are housed 4 per cage in and given sterilized laboratory chow and water ad *libitum* in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The animals are under permanent professional veterinarian care. Dr. Héctor Costa (MV) is our full time professional assistant, member of the CONICET (National Council of Scientific and Technological Research) Assistant Career, who takes care of the animal health. Besides, there are another 5 non-professional employees who take care of the different maintenance work (cleaning cages, feeding the animals, etc.) in the animal facility.

Computer:
Our Division counts with six computers, two of them connected to Internet. These computers are shared by 12 full-time investigators and fellows, and 3 part-time assistants. Our group has shared access to 2 of the computers (one with Internet connection) and only one is for exclusive use of our group (no Internet connection available)

Office:
Research fellows and assistants (8 in total) share a big room with 6 desks and 3 computers. The 6 independent investigators share 4 offices, 3 of them with computers .

Other:

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.
Our lab counts with 3 horizontal electrophoretic aparattus and a vertical one. Two table centrifuges (one for Eppendorff tubes, the other one for cells, both refrigerated), a hybridization oven, automatic developer, 2 PCR machines, 2 optic microscopes, a shaker for bacteria culture, 2 incubators for cell culture, 3 laminal flows, 4 refrigerators, three -20 freezers, one -80 freezer, a liquid nitrogen tank and an autoclave. All this equipment is fully available for our use at any time.

In other Sections of the National Academy of Medicine (same building, different laboratories), there are a Flow Cytometer and a Automatic sguencer that are available for our use with previous reservation.

8. RESEARCH PLAN

Tumor progression and apoptosis in mouse mammary gland

a) Specific aims:

What is the cause for the lack of response to the regulatory mechanisms of proliferation and death control observed in the neoplastic cells? This is the main question that experimental oncology addresses over and over again. In the mammary gland, hormones and growth factors associated to pregnancy, lactation and involution are the responsible for controlling mammary epithelium proliferation, differentiation and death. Understanding how the progressive alteration of the control exerted by these factors is involved in the process of mammary tumor initiation and progression is the long-term goal of our research.

The project presented below is focused in two main objectives:

A)Investigating mechanisms of progression of pregnancy-dependent mammary tumors

We count on a new mouse mammary tumor model for studying hormone-dependent mammary tumor progression that allow us to dissect the process quite meticulously. We are particularly interested in the progression of pregnancy-dependent tumors that remaine dormant in virgin females for long periods. Our preliminar results suggest that during dormancy, events that result in the selection of more aggressive and undifferentiated tumor cells are ocurring. This issue is very relevant from the scientific as weil as from the clinical point of view. However, there are not many experimental models that deal with this problem. Therefore, our approach could render some interesting insights into this field.

As explained below, the MMTV-induced mammary tumor models allow the identification of genetic loci that have been mutated during carcinogenesis . In our model we can identify mutations specifically associated with to progression of hormone-dependent cells to a hormone-independent phenotype. In addition, we set up a new approach for isolating and cloning these loci that will allow a faster discovery of sequences involved in this process.

B) Mammary apoptotic signals and tumor escape mechanisms

The results we have obtained investigating the role of the Leukemia Inhibitory Factor (LIF) on mammary gland development got us interested in the process of mammary gland involution. Right after weaning, apoptosis is triggered in many lobulo-alveolar mammary cells. Interestingly, the very early causes that determine the initiation of this process remain unknown. We then decided that investigating this early events could lead us to very seminal information about how apoptosis can be triggered in mammary epithelium. Therefore, we could find out whether those mechanisms are altered in mammary tumor cells. To achieve this goal we decide to 1) get a deeper knowledge about LIF role during mammary involution; 2) investigate the events that precede and proceed LIF expression and action; and 3) compare the data obtained in normal epithelium with mammary tumor cells.

Background and significance:

Mouse Mammary Tumor Virus-induced pregnancy dependent tumors. Origin and progression

Mouse mammary tumor virus or MMTV is a type B retrovirus causing tumors in susceptible mice by acting as an insertional mutagen in somatic cellular genes. MMTV is transmitted either horizontally as milk-born exogenous variants or vertically as germ-line integrated provirus (Mtv). This phenomenon has provided the basis for an approach to identify genes which, when affected, may contribute to cancer progression. Eight different genes have been shown to be genetically altered in multiple mammary tumors as a consequence of MMTV integration (Callahan, 1996).

Mammary tumors induced by exogenous MMTV frequently arise from preneoplastic lesions termed hyperplastic alveolar nodules (HANs). HANs can be serially transplanted in female mouse hosts. Upon transplantation these lesions originate pre-neoplastic clonal dominant cell populations from which, eventually, clonal-dominant mammary carcinomas and even metastases arise (Callahan & Smith, 2000). In consequence, it has been proposed that new MMTV insertions in cell genome followed by clonal selection is the main mechanism by which this tumor progression sequence proceeds.

Alternatively, some MMTV strains are able to induce premalignant ductal lesions named plaques, which arise during pregnancy and regress after parturition (Callahan, 1996). Plaques have also been serially transplanted and it was found that upon this procedure, these lesions retain the ability to produce hormone-dependent (HD) tumors in pregnant females while they grow into normal ducts in non-pregnant hosts (Aidells & Daniels, 1974). It was shown that these HD tumor lines consist mainly of clonal dominant populations (Peters et al, 1984). However, other authors have reported that early passages of HD tumors from GR mice have different mutated loci than later passages showing hormone-independent (HI) behavior (Michalides et al., 1982). This suggested the possibility that the HD tumors were composed of polyclonal populations

It has been previously shown that identification of MMTV insertions by Southern blot analysis in a mammary cell population indicates their clonal or nearly clonal origin (Kordon & Smith, 1998). Unique virus-host restriction fragments constitute a specific and reproducible pattern of bands only if they are present in most cells in a population. In case this was derived from the expansion of many different progenitor cells, then specific MMTV-host restriction fragments would be hard to detect, since retroviral DNA insertions occur randomly at multiple sites in cellular DNA (Withers-Ward et al., 1994). In fact, it was shown that intact lactating MMTV-infected mammary glands which represent polyclonal populations -because secretory lobules develop from multiple progenitors at multiple sites- do not show a pattern of specific MMTV-host restriction fragments (Kordon & Smith, 1998).

Three new exogenous MMTV variants (BALB2, BALB14 and LA MMTV) were described in our mouse colony (Golovkina et al., 1997; Buggiano et al., 1999). The three variants appearing together were designated MMTV (LA) in recent publications (Golovkina et al., 1997; Buggiano et al., 2001). BALB/c infected with MMTV (LA) showed a 90% incidence of mammary tumors. These tumors were initially pregnancy-dependent. They regressed completely or partially between pregnancies and they re-appeared at the same site in subsequent pregnancies. Eventually, they could progress to become autonomous and grow independently of the female hormonal status (Buggiano et al., 1999).

Leukemia inhibitory factor involvement in mammary gland biology

The pleiotropic cytokine leukemia inhibitory factor (LIF) is a secreted 38-67 KDa glycoprotein first named for its ability to induce macrophage differentiation in the murine myeloid leukemic cell line

M1 (Gearing et al., 1987). Messenger RNA for LIF has been detected in a variety of adult mouse tissues and displays different biological activities including effects on bone metabolism, inflammation, neural development, embryogenesis and the maintenance of implantation (Taga et al., 1997; Hilton et al., 1994). In the mouse uterus, LIF is expressed in the endometrial glands coincidentally with the time of blastocyst implantation. Females that lack a functional LIF gene are fertile, but their blastocysts are unable to implant in uterus (Stewart et al., 1992). In the uterus, LIF expression occurs possibly as a direct response to the increase of circulating levels of estrogen occurring on day 3 or 4 of pregnancy, coincidentally with implantation. Out of pregnancy, during the menstrual cycle, it has been shown that LIF expression in the uterus is confined to the glandular epithelium during the mid and late secretory phases (Cullinan et al., 1996; Shen et al., 1992).

A potential role for LIF in the pathogenesis of human breast cancer was indicated by the finding of LIF protein production by the MDA-MB 231 breast cancer cells (Kellokumpu et al., 1996). In addition, the increase on proliferation that this factor induces on several estrogen-dependent (MCF-7 and T47D) and estrogen-independent (SK-BR3 and BT20) breast cancer cell lines and fresh breast carcinoma cells, indicates that LIF would play an important role in mammary neoplastic cell replication (Kellokumpu et al., 1996; Estrov et al., 1995). More recently, it has been found that LIF expression in these cells can be modulated by progestins and antiprogestins (Bamberg et al., 1998). However, we have not found in the literature any evidence reporting LIF expression and/or activities in the normal mammary gland tissue. Therefore, we decided to investigate whether LIF is differentially expressed in the mammary gland developmental stages and whether this protein actively participates in the mammary epithelium growth and/or function. The fundamental role that estrogen and progesterone play on mammary gland proliferation and differentiation made us hypothesizes that LIF could play a relevant role in these processes in either virgin or pregnant females. However, our results show that LIF expression is up-regulated during post-lactational involution, being associated to the secretory epithelium apoptosis that occurs at the early phase of this period.

Reports of LIF biological activity on breast cancer cells also differs from our observations in the normal mammary gland. It has been shown that LIF stimulates cell growth in several breast cancer cell lines in a dose dependent manner (Estrov et al., 1995, Kellokumpu et al., 1996). However, our results show that *in vivo*, LIF expression is associated with natural occurring cell death and that exogenous LIF treatment induces apoptosis in the mammary gland. In addition, we have found that proliferation of a normal mouse mammary epithelial cell line, NMuMG, is significantly inhibited in a dose-dependent manner by LIF treatment. More experiments are required to determine whether these differences could be due to the neoplastic vs. normal, or the human vs. mouse nature of the tested mammary cells.

The involution of mammary gland following weaning involves a set of events that results in returning to a virgin-like state. This process involves two distinct stages. The initial one is characterized by programmed cell death of the differentiated epithelial cells and induced expression of apoptosis related proteins (Lund et al., 1996, Li et al., 1997 and Nguyen et al., 2000). This is followed by a second stage that involves extensive tissue remodeling and a characteristic spatial and temporal expression pattern of a number of extracellular proteinases (Lund et al., 1996). The LIF expression pattern shown by our results indicates that this factor is associated with the first stage of mammary gland involution. Its involvement in this phase is supported by the fact that LIF pellet implantation in lactating mammary glands induced a significant increase of epithelium apoptosis, while no effect on the mammary architecture was observed. It has been previously found that first stage of involution is dependent upon mammary-derived local signals induced as a consequence of milk-stasis, while the second one is caused by the loss of circulating lactogenic hormones (Nguyen et al., 2000). These facts

fit well with our data showing that interruption of milk efflux during lactation increased LIF expression levels significanly. Alternatively, no major changes in LIF expression were noted when a lactogenic hormone as Hyd was inoculated in mouse females during mammary involution. The specific signals that derive from milk stasis and result in local factor, like LIF, release remain unknown. It has been postulated that cellular stretching associated with milk accumulation in the alveolar lumina could be responsible for STAT3 activation (Chapman et al., 1999) as previously reported for cardiomyocytes (Pan et al., 1999). Similarly, LIF expression was induced in cardiac miocytes in *ex vivo* experiments of feline heart hemodynamic overloading (Wang et al., 2001). Although it is a very interesting possibility, more experiments need to done in order to address the question whether cellular stretching could be fully responsible for the release of LIF and other local factors during the first phase of mammary involution.

Apoptosis is an intrinsic part of mammary involution. Natural weaning as well as litter removal stimulates oligonucleosomal DNA laddering and changes in gene expression indicative of apoptosis. Interestingly, similarities in the activation of signaling cascades and gene expression patterns were observed between the first stage of mammary involution and the few experimental models in which LIF induced programmed cell death.. Apoptosis during involution was associated to STAT3 activation (Li et al., 1997), expression of cell cycle control proteins as c-Jun, JunB; JunD, c-Fos and c-Myc, (Marti et al., 1994) and death related genes as Interleukin 1b Converting Enzyme (ICE) (Lund et al., 1996) or Bax (Heermeier et al., 1996; Quarri et al., 1996). On the other hand, it has been reported that LIF activates terminal differentiation and apoptosis in myeloid leukemia M1 cells through STAT3 activation (Minami et al, 1996); this factor induced apoptosis of cultured sympathetic neurons associated to Bax and c-Jun N-terminal kinase (JUNK) (Savitz & Kessler, 2000), and LIF induced growth arrest and increased cell death rate in two carcinoma cell lines associated to c-Myc and ICE expression induction (Kamohara et al., 1997). Therefore, the activation of these common signaling pathways can be indicating possible mechanisms for LIF apoptosis induction in the mammary epithelium.

The participation of activated STAT3 in mammary gland involution has been already demonstrated. Mammary local factors stimulate STAT3 phosphorylation during involution (Li et al., 1997) and mammary gland of STAT3 conditional knock-out mice showed suppression of epithelial apoptosis that led to a dramatic delay in mammary gland involution (Chapman et al., 1999). Our results show an increase in the level of STAT3 phosphorylation in the LIF treated mammary gland, in which apoptosis induction was also shown. Therefore, this suggests that LIF might induce mammary epithelium programmed cell death by activation of the STAT3 signaling pathway.

c. Preliminary studies

MMTV(LA) induced mammary tumor behavior

First transplant generation

Seventeen primary tumors from pregnant MMTV (LA)-infected females were transplanted in inbred uninfected BALB/c female mice. We found that most of them (11/17) were fully able to develop in female hosts when impregnated, but did not show any growth for more than 5 weeks when transplanted into virgin hosts. Tumor lines that showed this behavior were called pregnancy-dependent or hormone-dependent (HD) tumors. Figure 1 shows that some of these HD lines (n=6) showed regression after each parturition (Figure 1a, Tumor D-2A), while others did not (n=5) (Figure 1b, Tumor 2236).

Alternatively, some of the primary tumors were able to grow in virgin hosts. However, some of these lines (n=4) were still able to respond to pregnancy since tumor transplants grew earlier and faster in multiparous females than in virgin hosts (Figure 1c, Tumor 2216). We refer to them as pregnancy or hormone-responsive (HR) tumor lines. Only 2 of the primary tumor transplants grew in virgin hosts and their development was not stimulated by pregnancy (Figure 1d, Tumor 2126); these are referred to as pregnancy-independent or hormone-independent (HI) tumor lines.

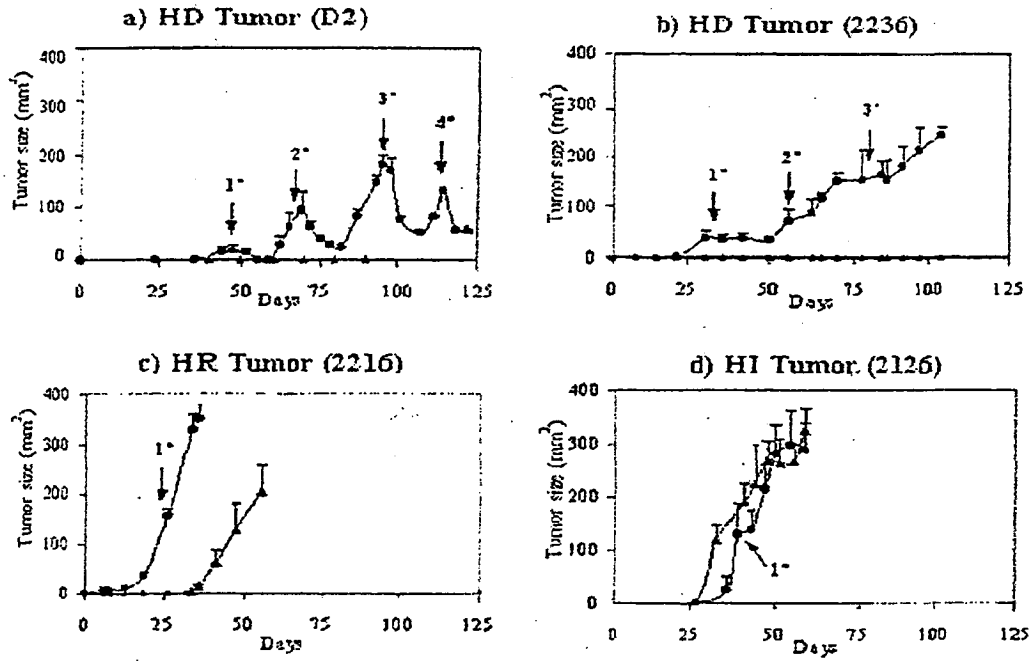

Figure 1. Examples of the four different tumor growth patterns at first transplant generation: a)Hormone-dependent (HD) tumor showing regression after each pregnancy, b) HD tumor which does not regress after each pregnancy, c) Hormone-responsive (HR) tumor, d) Hormone-independent (HI) tumor. Each point represents the mean surface of 3 to 8 mammary tumors passages + SD in multiparous (●) and (▲) virgin females. The arrows indicate the end of each pregnancy.

Figure 2 shows Southern blot analysis of multiple first passages from 6 primary tumors (4 HD, 1 HR and 1 HI). Only in a few cases, bands associated with MMTV insertions were clearly observed in all first transplant generation implants from an HD tumor. Figure 2B shows one of these cases in which all the passages showed clear additional bands corresponding to exogenous MMTV insertion events occurring in most tumor cells. On the other hand, Figure 2C shows that most implants from HD tumors did not show a stable pattern of bands. In tumor 2144, bands corresponding to exogenous MMTV are observed in only one implant, while no exogenous insertions are detectable in the other 6 parallel transplants. Tumor 2280 shows two bands in one of the transplants, but only one of these bands is present in a second implant and possibly in the third one, but with lower intensity. In the case of tumor 2236 a single band corresponding to exogenous MMTV insertions was found in only two of the three assayed DNA samples. Figure 2D shows the band patterns corresponding to 2216 primary tumor and its successive passages. This tumor line showed an HR behavior. Once again, a stable pattern of bands was not found in the first transplant generation implants. Only one of these three implants showed an identical pattern to the one found in the primary tumor. Other implant showed an extra band, while the last one showed one band less. The fact that one of the tumor passages showed fewer bands than the primary tumor strongly suggests that the latter was composed of several cell populations. Eventually, one of these populations became predominant and gave rise to a clonal or quasi-clonal population as seen in passage 2 and 3.

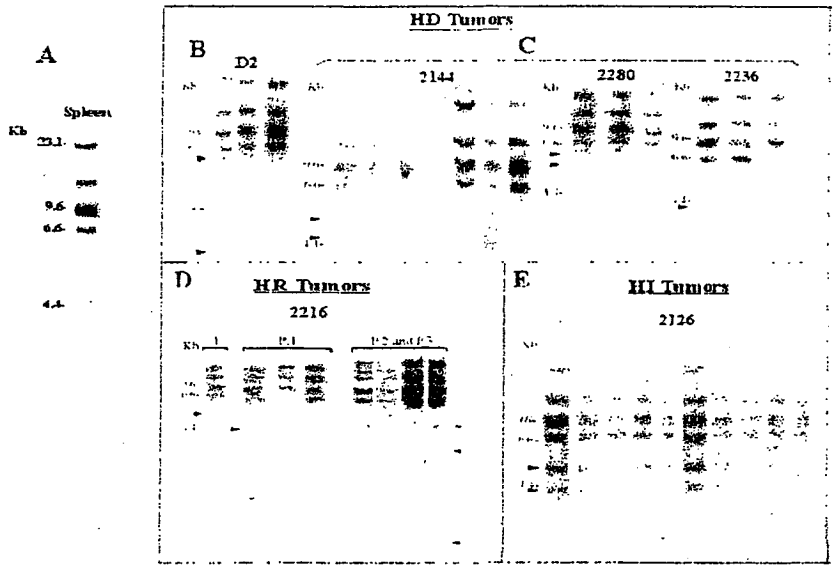

Figure 2. Southern blot analysis of Eco RI digested DNA from tumor transplants with different growth patterns:
(A) spleen DNA digestion pattern from a BALB/c mouse is displayed in order to despict Eco RI restriction pattern of endogenous MMTV sequences. B) & C) Four HD tumors at first transplant generation; D) HR, primary tumor (1°), first (P1), second and third (P2 and P3) transplant generation; E) HI tumor, first transplant generation. DNA from each tumor was digested with Eco RI, and Southern blot analysis was carried out. In every case the blots were hybridized with a specific probe for MMTV-LTR sequences. The arrowheads indicate the location of host-viral restriction fragments indicative of the presence of exogenous MMTV proviral insertion within the somatic DNA. The 5 upper bands correspond to endogenous MMTV sequences present in BALB/c mouse genome.

When no exogenous MMTV associated bands were detected in Eco RI digested tumor DNA, it was possible that they were masked by bands corresponding to similar size fragments generated from

endogenous MMTV-LTR sequences. Therefore, in those cases, other restriction enzymes such as Hind III and Bgl II were used in an attempt to unmask putative hidden bands. However, analysis of HD first generation transplants, which did not show new insertion bands after EcoRI digestion, also failed to provide evidence of a homogeneously mutated tumor cell population when treated with other enzymes (data not shown).

Passages from the HI tumor lines showed a very strong and stable pattern of bands corresponding to exogenous MMTV cDNA insertions, indicating that these tumors were mostly clonal derived cell populations. An example of this, in which the 10 parallel transplants from HI tumor 2126 displayed the same two extra bands, is shown in Figure 2E. Therefore, these results suggest that while MMTV (LA) tumors showing an HI pattern of growth are mostly clonal derived cell populations, pregnancy dependent lesions can be composed of several different cell populations, each of them with different MMTV(LA) insertion sites.

Pregnancy-dependent tumor progression

After consecutive passages, HD and HR tumors progressed to an HI behavior. We found that selection of hormone-independent cells by a hormone-deprived environment was not necessary for the progression of these tumor variants. This was observed in at least 4 different HD tumor lines. For example, Figure 3 shows how D2 HI tumor variants appeared very early after implantation. In the first transplant generation, they appeared before and, in the second one, during the first pregnancy. SBA showed that in both of these HI lines there were extra bands, not present in the HD passages of this tumor line. These bands were observed in all parallel and subsequent passages of these HI variants. Interestingly, although they appeared independently, both D2 HI variants showed the same pattern of bands suggesting that they originated from the same cell subpopulation already present at a low percentage in the HD tumor.

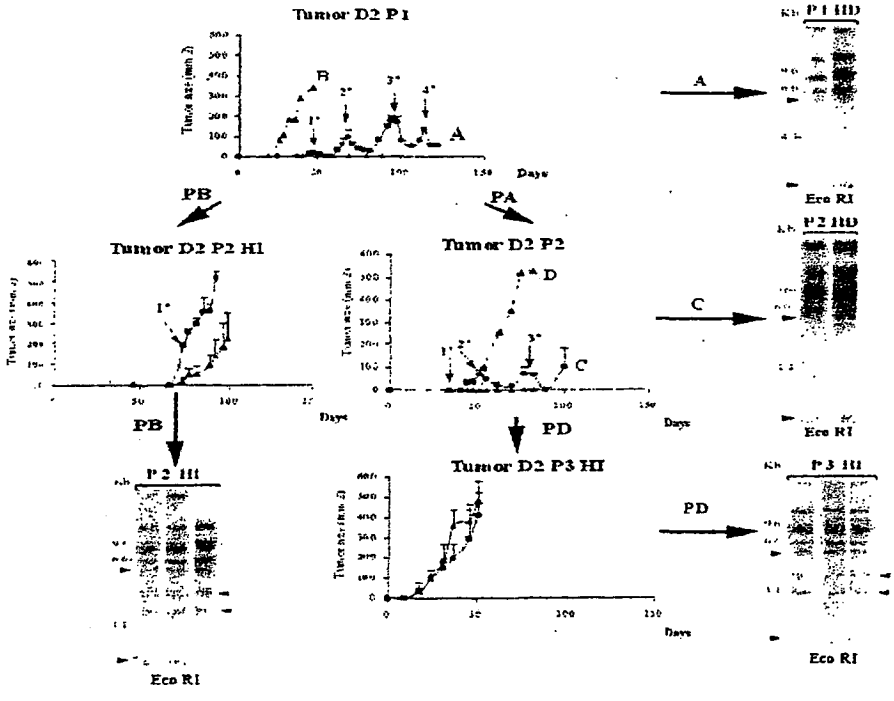

Figure 3 Tumor D2 Progression to hormone-independence

P1: First transplant generation, P2: Second transplant generation, P3: Third transplant generation. A) HD first transplant generation (n=6), B) HI first transplant generation (n=1), C) HD second transplant generation (n=5), D) HI second transplant generation (n=1). PA: passages of tumors A, PB: passages of tumor B, PD: passages of tumor D. In tumor growth curves (when n>1) each point represent Media ± SD of 4-6 tumor transplants in multiparous (●) and (▲) virgin females. Small arrows indicates tumor passages. Large arrows indicate Southern blot analysis of A, C, PB and PD tumors. Tumor DNA was digested with Eco RI and hybridized with a probe specific for MMTV-LTR sequences. Arrowheads show exogenous MMTV proviral insertion sites.

Figure 4 shows other examples of tumor progression patterns. HD tumor passages that remained dormant for more than 4 months in virgin females, would grow during a late pregnancy or upon hormone-stimulation. However, they behaved as HI tumors in the following generation. This pattern of progression was observed in 6 different HD tumor lines (Figure 4A shows one of them).

In several cases, HD tumors progressed slowly to an HI phenotype, through several HD and/or HR passages. Tumor 2280 is an example of this. In this case, bands by SBA showing the predominance of a clonal subpopulation were already observed at passage 3, which showed an HR behavior (Figure 4B).

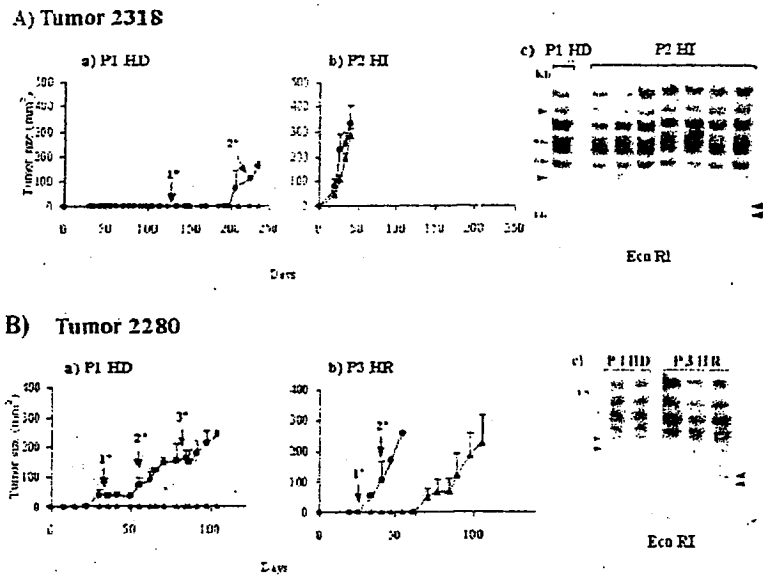

Figure 4 Progression to hormone-independence.
(A) Tumor 2318 as an example of tumors that progressed following a dormant state. (B) Tumor 2280 as an example of HD tumors that progressed through HR stage/s.
a) Growth at first transplant generation, b) Growth at a second transplant generation. Each point represents the mean The arrows indicate the end of each pregnancy. c) Southern blot analysis shows restriction pattern of MMTV proviral insertions. Arrowheads show exogenous MMTV viral insertions. P1 HD: hormone-dependent first transplant generation; P2 HI: hormone-independent second transplant generation; P3 HR: hormone-responsive third transplant generation. In panel showing

We have found that tumors that went through a dormancy period (150 to 300 days) only grew upon hormone stimulation. Nevertheless, they showed a HI pattern of growth in the successive passages. Interestingly, they also showed specific histological features: high level of fibrosis and squamous metaplasia foci that suggest the presence of progenitor epithelial cells that went through diverge differentiation towards ectoderm. None of these features were common in tumors that did not go through dormancy.

color prints in the appendix

Figure 5 : Hematoxilin-Eosin staining of tumor passages that remained dormant in virgin females and resumed grow upon hormone stimulation. It is noticeable the high amount of fibrosis and the presence of squamous metaplasia foci (insert) in the transplants.

Tumor progression. Histological and ER & PR content analysis.

At first transplant generation, all the HD and HR analyzed tumors were well-differentiated mammary adenocarcinomas with an either papillary-cystic or ductulo-acinar morphological pattern. Alternatively, HI tumors showed a variety of histological patterns, while some HI tumor lines resembled the pattern found in HD carcinomas, others showed a very poorly differentiated architecture. Figure 6 shows examples of 3 different patterns found in our mammary tumor passages. First, a ductal-cystical papillary pattern commonly found in HD transplants is displayed (A). The second panel (Figure 6B). shows a well-differentiated glandular-cystic morphology found in an HR passage. Finally, a poorly differentiated adenocarcinoma is shown in Figure 6C, corresponding to an HI tumor_line. Noteworthy, scattered areas with the latter morphology comprised from 5 to 40% in HR tumors and up to 90% in HI ones.

Figure 6. Histological features of MMTV-induced mammary tumors progressing from HD to HI phenotype.
(A)Well differentiated adenocarcinomas with papillary formations into lumen of cystic ducts and stromal invasion that were mostly found in HD tumors. (B) Adenocarcinomas with cystic and solid- cribiform pattern that were seen most commonly in HR tumors. (C) Poorly differentiated adenocarcinomas with predominant solid pattern were found in several HI tumors (H&E, 125X).

A very good correlation was found between hormone-responsiveness and the expression of ER and PR. Immunohistochemical analysis showed high number of cells expressing these receptors in all the analyzed HD and HR tumor transplants (Table 1; Figure 7 A & C). However, HI tumors -even the well-differentiated ones- always presented low ER and PR levels (Table 1, Figure 7 B & D). Therefore,

it can be concluded that progression to autonomous growth was associated with a significant loss of ER and PR content. Similar results were obtained when ER and PR content was analyzed by Western blot analysis: HD tumors showed higher ER and PR expression levels when compared with HI passages. Representative Western blots are shown in Figure 7 E. color prints in the appendix

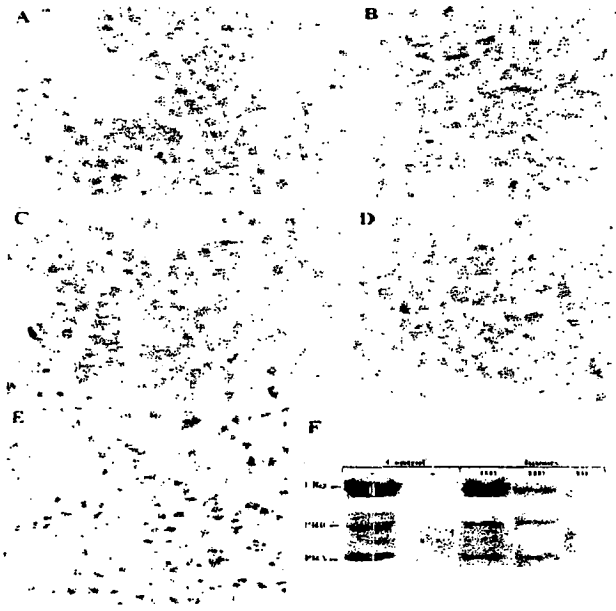

Figure 7: Immunostaining for Estrogen (ER) and Progesterone Receptor (PR).
In HD tumors (*e. g.* Tumor 2228 IID P1), numerous nuclei were strongly stained for ER (A) and PR (C shows two regions in the same sample). In HI tumors (*e. g.* Tumor D2 HI P2), few nuclei were weakly labelled for ER (B) and almost none was positive for PR (D) (DAB, 600X). (E) Negative control: Immunostaining of an HD tumor (Tumor 2228 HD P1) in which policlonal rabbit antibodies that recognize ER or PR were replaced by normal rabbit serum (DAB, 400X). (F) Western blot of PR isoforms (PR A, M_r 83,000; PR B, M_r 115,000) and ERα in two HD tumors (Tumor D2 HD, P2 and Tumor 2314 HD P1) and an HI tumor (Tumor 2216 HI P5). Similar bands were obtained in 5 different tested HD tumors; no bands were detected in 4 different tested III tumors. Uteri obtained from mice with 10 μg/kg E_2, were used as positive control (Control +) and mouse muscle tissue as negative control (Control -).

Table 1. Immunostaining for estrogen and progesterone receptors in HD and HI tumor transplants.

	ER (% positive nuclei ± SD)	**PR (% positive nuclei ± SD)**
HD passages (n=7)	**55.40* ± 16.7**	**48.45* ± 9.99**
HI passages (n=5)	**10.30 ± 2.1**	**11.53 ± 4.7**

*HD tumor passages showed a significant higher percentage of positive nuclei for ER and PR by Student's two-tailed *t*-test ($p<10^{-3}$).

Cloning and sequencing of MMTV(LA) insertion sites.

In order to identify genome sequences altered by MMTV (LA) insertions in our mouse tumor model, the technique known as Inverse-PCR was performed. This procedure has been successfully used for different purposes (Ochman et al, 1988; Sheng-He H, 1994; Willis et al., 1997, Li et al., 1999), but its utilization for identification of MMTV insertion sites has not been reported. At the present time, in our model, insertion sites from 3 different tumor lines have been already analyzed.

Briefly, the following procedure has been followed. Tumor DNA (1μg) was digested to completion with EcoRI in a total volume of 30 μl. Reactions were stopped by heating at 70° C for 10

min. Digested DNA was then self-circularized by dilution and ligation using T4 DNA ligase in a total volume of 600 ul overnigth at 16° C. Circular DNA was precipitated with ethanol and dissolved in 10ml of water. Two µl of this dilution were used in a 50ul PCR reaction containing dNTPs, forward and reverse MMTV (LA) specific primers, 1x buffer B and enzyme mix in the E-Longase PCR System (Gibco-Life technologies). We tailored IPCR conditions so as to make it possible to amplify fragments as large as 12Kb.We used a thermocycler programmed as 92°C 2 min, followed by 30 cycles of 92°C 30 seg., 60°C 30 seg., 68°C 10 min., then a final extension step at 68°C for 15 min. The amount of PCR product was quantified by electrophoresis on a 1% agarose gel. The PCR products were purified and directly cloned in the pGem T Easy vector system (Promega). The primers used for I-PCR reaction are highly specific for MMTV (LA) exogenous virus and do not hybridize with MMTV-endogenous sequences of BALB/C mice. For sequencing, the same or downstream non-specific MMTV-LTR primers were used. DNA sequencing was performed using the PRISM BigDye Cycler Sequencing kit on ABI Model 373A DNA Sequencer. Figure 8 shows a diagram of the I-PCR procedure.

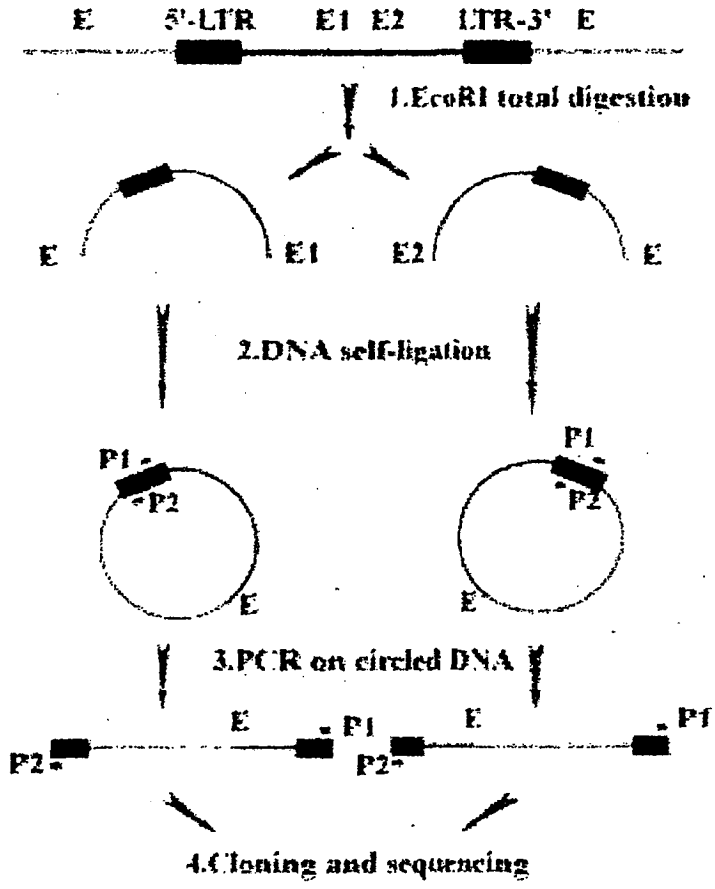

Figure 8. Inverted PCR (IPCR) procedure. Small arrowheads indicated localization of the amplification primers

The following figure (Fig.9) shows the Southern blot analysis of tumors D2-HI (A), 2284-HI (B) and 2312-HI (C), and the corresponding fragments obtained by I-PCR. After cloning and sequencing of those clones it was found that the three cases corresponded to INT2/Fgf3 locus insertions (Figure 9 D).

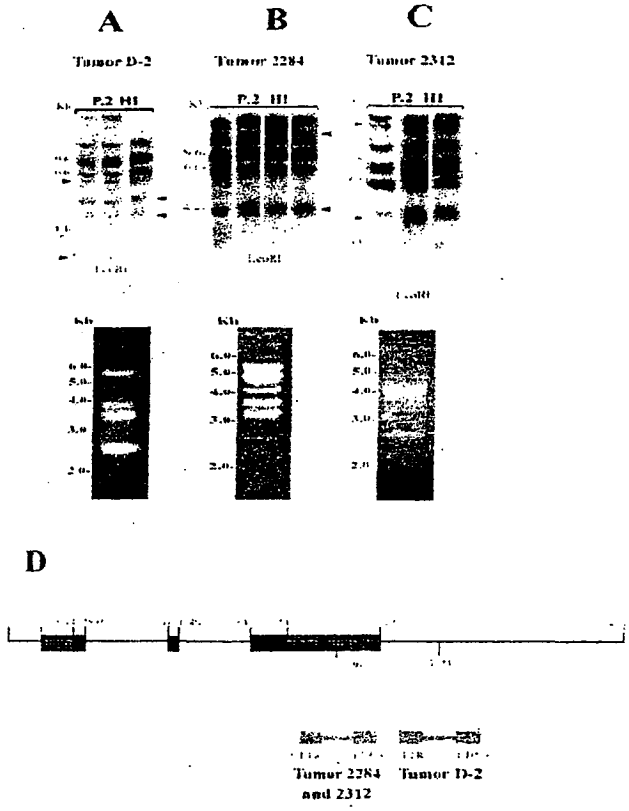

Figure 9. Southerns blot analysis of EcoRI digested DNA from HI tumors of second passage and IPCR product of A) D-2, B) 2284 and C) 2312 tumor lines. D) Int-2 gene. Coding and no-coding regions are indicated. Black lines indicated intron portion.

Leukemia Inhibitory Factor (LIF) involvement in mouse mammary gland development and involution.

LIF expression pattern during different developmental stages of the mouse mammary gland

Relative levels of LIF gene expression during mammary development were examined at several different stages of growth, differentiation and involution. Mammary glands from virgin females with rapidly proliferating ducts (33 days old), adult virgin female with growth quiescent mammary glands (80 days old), pregnant females during lobule-alveolar proliferation and differentiation, nursing females with fully differentiated glands, and females 24 hours after weaning when many of the differentiated cells are dying, were assayed for mouse LIF expression by semi-quantitative RT-PCR (Fig 10 A). The results show that LIF is expressed at detectable and similar levels in puber, adult virgin and pregnant females. However, LIF mRNA dropped to almost undetectable levels right after parturition and remained low during the whole lactation period. LIF expression levels did not show significant fluctuations among the different phases of each of these developmental stages: No

differences were found among different phases of the estrous cycle in virgin females; 1st, 2nd and 3rd week of pregnancy; and 1st, 2nd and 3rd week of lactation (data not shown). On the other hand, 24 hours after weaning, LIF mRNA levels showed a steep increase, being this level of expression significantly higher, not only with respect to lactation, but also to adult mammary glands (Fig 10A). LIF expression was also tested in epithelium-divested mammary glands (cleared #4 fat pads) of adult females at different stages of the estrous cycle and at different times during pregnancy. Mammary fat pads of females that were ovariectomized at the third week of age were also tested. In all these cases low, but detectable levels of LIF expression in the mammary stroma were detected. Once again, no variations in LIF mRNA levels were found related to hormone status either in virgin or pregnant females. Due to the lack of LIF level variation between these conditions, the data referring to LIF stromal expression were pooled and the average level is shown in the corresponding column of Fig 10A. These results show that at least part of the mammary LIF expression found in virgin and pregnant females could be due to transcription occurring in stromal mammary cells.

In order to confirm the up-regulation found during involution, and to check LIF transcript size, Northern blot analysis, using polyA RNA from adult virgin, lactating and involuting mammary glands, was carried out. High levels of a unique 4.2 Kb. LIF transcript were found only at involution phase. By this method, LIF expression was undetectable in mammary glands of both nursing and virgin adult females (Fig. 10B).

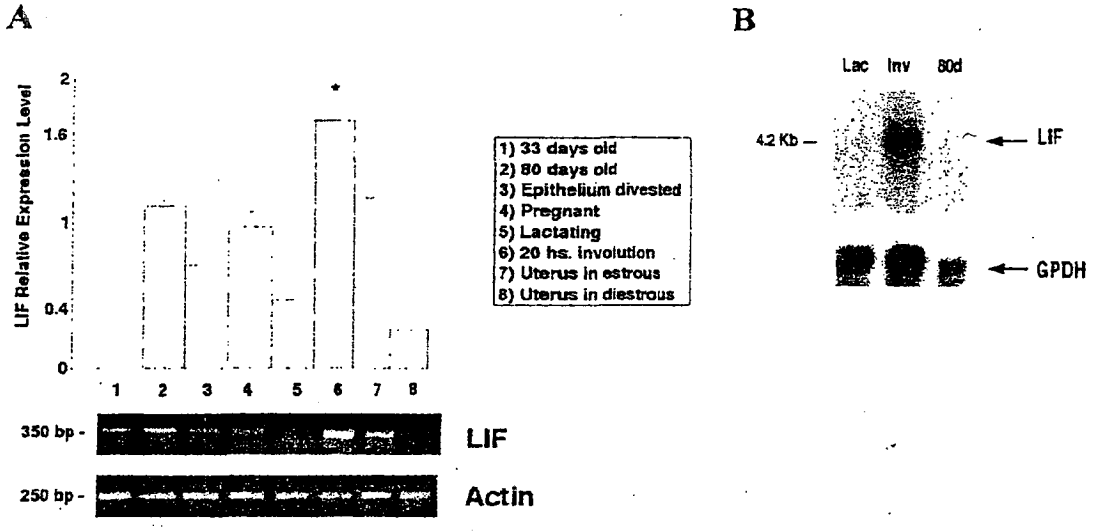

Figure 10: LIF expression levels at different stages during mammary gland development and differentiation. A) LIF expression levels as determined by RT-PCR using mouse mammary RNA (lanes 1-6) from: 33 days old puber virgin females (lane 1), 80 days old adult virgin females (lane 2), epithelium divested glands (lane 3:), pregnant females (lane 4), nursing females (lane 5), 20 hours after weaning (lane 6) and mouse uterus (lanes 7 & 8), in estrous (lane 7) and diestrous (lane 8). Upper panel: Semi-quantitative comparison of LIF expression levels. Results are presented as a ratio of that detected in total mammary RNA from 33 days old virgins (fixed value=1). Each column represents the mean, and bars represent the SE, determined from 4 to 6 separate RT-PCR experiments. * indicates a significant increase in the relative LIF expression level when the involuting gland is compared with the adult virgin 80 days old female mammary glands ($P<0.01$). Lower panel: ethidium bromide stained gel showing one of the RT-PCR assays described above. B) Northern blot analysis of LIF expression at different mammary gland stages. Six μg of poly-A RNA from lactating (Lact.), involuting (Inv.) and 80 days old virgin female mice (80d) were loaded in each lane and hybridized with a LIF cDNA specific probe (upper panel) or a GPDH cDNA specific probe (lower panel).

Because of their higher sensitivity, RT-PCR semiquantitative assays were carried out in order to obtain a more detailed analysis of LIF expression during involution. These experiments revealed that during the first 8 hours of involution LIF levels remained low. However, 7 hours later (15 hours involution) a detectable increase was observed. These levels peaked 20 hours after the pups had been removed and lasted up to 3 days after weaning. Twenty-four hours later, LIF expression had recovered similar levels to those found in virgin females (Fig 11A).

Mammary LIF Receptor (LIF-R) expression levels at all the physiological conditions described above were also tested by semiquantitative RT-PCR analysis. In this case, no differences were found among virgin, lactating and different times of involuting glands (Fig. 11B). Thus, LIF expression regulation is not associated to the LIF-R levels.

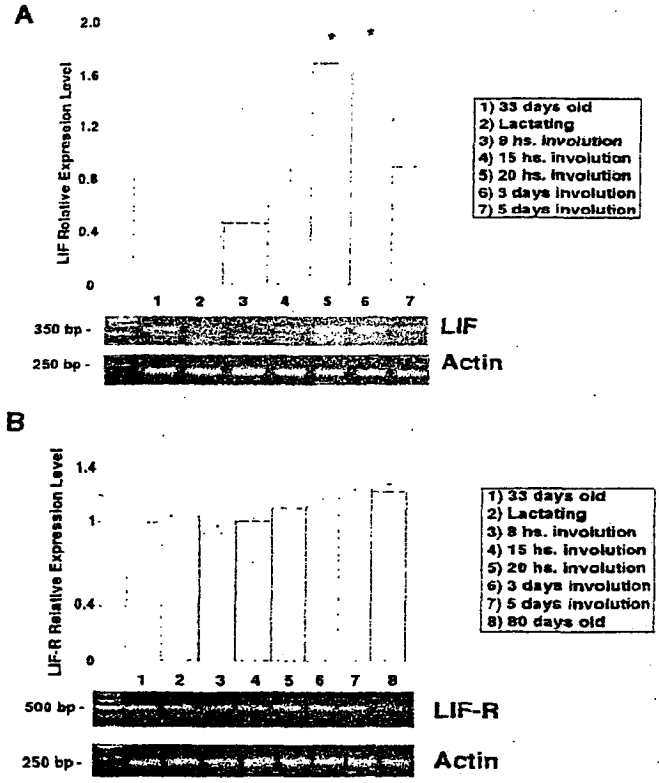

Figure 11: LIF and LIF-R expression levels at different stages during mammary gland involution.

A) LIF expression levels as determined by RT-PCR using mouse mammary RNA from: 33 days old virgin females (lane 1), nursing females (lane 2), 8 hours (lane 3), 15 hours (lane 4), 20 hours (lane 5), 3 days (lane 6) and 5 days (lane 7) after litter removal. Upper panel: Semi-quantitative comparison of LIF expression levels. Results are presented as a ratio of that detected in total mammary RNA from 33 days old virgins (fixed value=1). Each column represents the mean, and bars represent the SE, determined from 4 to 6 separate RT-PCR experiments. * indicates a significant increase in the relative LIF expression level of mammary glands at both 20 hours and 3 days after weaning, compared with the level found in 5 days of involution ($P<0.01$). Lower panel: ethidium bromide stained gel showing one of the RT-PCR assays described above. B) LIF-R expression levels as determined by RT-PCR using mouse mammary RNA from: 33 days old virgin females (lane 1), nursing females (lane 2), 8 hours (lane 3), 15 hours (lane 4), 20 hours (lane 5), 3 days (lane 6) and 5 days (lane 7) after litter removal, and 80 days old virgin female mice (lane 8). Upper panel: Semi-quantitative comparison of LIF-R expression levels. Results are presented as a ratio of that detected in total mammary RNA from 33 days old virgins (fixed value=1). Each column represents the mean, and bars represent the SE, determined from 4 to 6 separate RT-PCR experiments. Lower panel: ethidium bromide stained gel showing one of the RT-PCR assays described above.

Immunohistochemical analysis of LIF expression also revealed its increase at the protein level during the first stage of mammary gland involution. Positive staining was found in the cytoplasms of epithelial cells as well as in the intercellular spaces of regressing lobules and ductules. On the other hand, no staining was found in the mammary epithelium of lactating mice (Figure 12). We have also observed scattered positive LIF stained epithelial nuclei in the 48 and 72 hours involuted glands.

color picture in the appendix

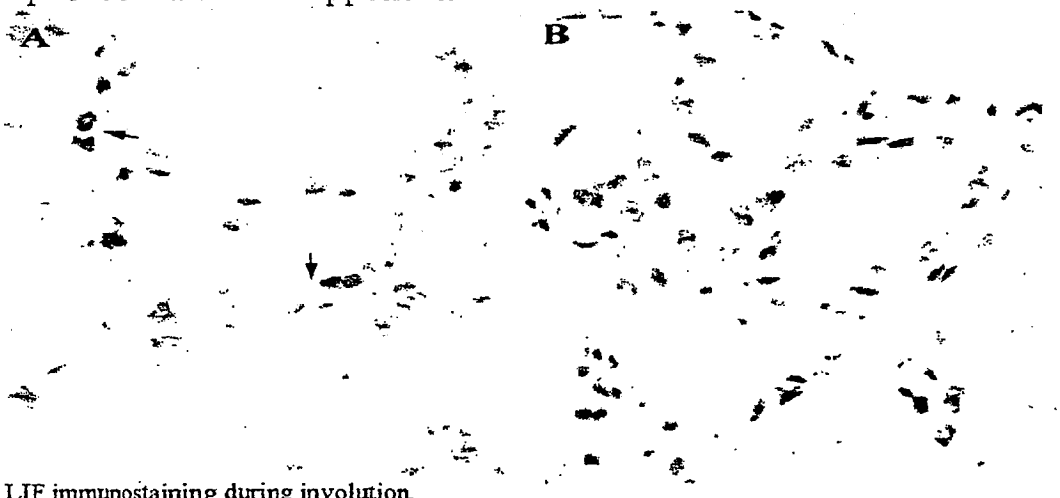

Figure 12: LIF immunostaining during involution.
Cytoplasmic LIF expression (arrows) was observed in epithelial cells at the 3rd day of mammary gland involution (A). However, not positive staining was detected if the first antibody was replaced by PBS in a similar tissue sample (B).

To determine whether other members of the LIF cytokine family would follow the same expression pattern, semiquantitative RT-PCR analysis was carried out with IL-6 specific primers. Although an increase in IL-6 expression was found associated with mammary involution, several facts suggested that IL-6 and LIF would not be under exactly the same regulation. Similar IL-6 expression levels were found in mammary glands from virgin, pregnant and lactating females, thus, no inhibition was associated with lactation (data not shown). In addition, IL-6 expression level in the mouse mammary gland required at least 40 amplifying cycles and 2 to 3 µg of RNA for amplifying IL-6 mRNA, while LIF expression needed a significant lower number of amplifying cycles and template amount (see Material and Methods). Alternatively, only 28 cycles and 1ug of RNA were required when testing IL-6 expression in dendritic cells or lymphocytes (using the same primers).

LIF expression is regulated locally by milk stasis

The pattern of LIF expression during involution indicated that it was associated to the first stage of this process. It has been demonstrated that local factors rather than systemic hormones regulate the events occurring during this phase (Liu *et al.*, 1996). In order to confirm whether LIF expression is triggered by the lack of milk efflux and the consequent release of local factors, one of the #4 mammary glands of lactating females was either sealed or its connection to the nipple surgically removed while the other 9 glands remained intact. In this way, lactogenic hormones maintained their systemic level while involution associated local factors were triggered in the sealed gland. The experimental mice continued nursing their pups for more than 3 days when both #4 glands were removed. Figure 13A shows that sealing as well as surgical procedures induced a significant LIF expression increase, although sealing the teat was more effective. In fact, the LIF increase resulting from this treatment was approximately the same as that observed in the involuting gland 72 hours after weaning. These results show that milk stasis would be enough stimulus to induce LIF transcription in mammary glands of females with a high level of circulating lactogenic hormones.

It was then decided to investigate whether lactogenic hormone treatment would be able to reduce LIF expression in the mammary gland after weaning. Treatment with glucocorticoids (Hyd) was chosen because these hormones have been reported to exert a negative effect on LIF expression in other experimental models (Bamberger et al., 1997). For this purpose, pups were removed from their mothers and half of them were treated with hydrocortisone (Hyd) for the 3 following days after which the mammary glands were examined. Hyd did not induce a significant inhibitory effect on mammary LIF expression during involution (Fig. 13A). This result confirms that the decrease of circulating glucocorticoid level during involution does not play a relevant role in inducing LIF expression after weaning.

Northern blot analysis was carried out to confirm the strong induction of LIF expression observed in sealed glands during lactation. By this method, similarly high expression levels were observed in mammary glands of non-treated females 3 days after weaning and in females with #4 sealed teats during 3 days in the midst of lactation. Contrarily, no LIF was detected in intact mammary glands from either late pregnant or nursing females (Fig. 13B).

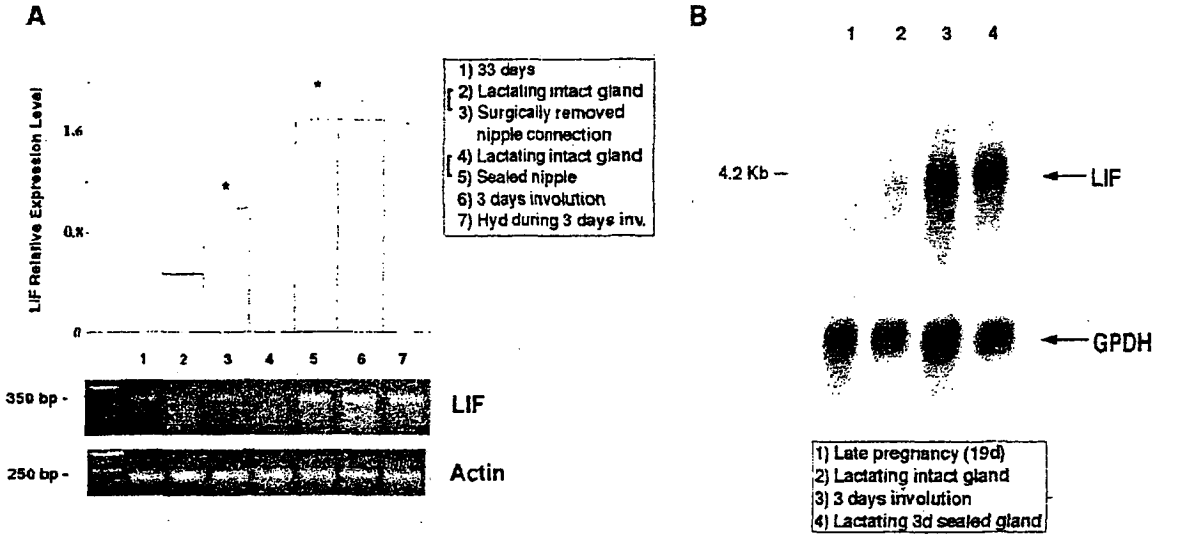

Figure 13: Effect of local vs. circulating factors on LIF expression induction.
A) LIF expression levels as determined by RT-PCR using mouse #4 mammary gland RNA from nursing females (lanes 2-5) in which the right glands were left intact (lanes 2 and 4) or milk efflux from the contralateral glands were eliminated either surgically (lane 3) or by sealing the nipple (lane 5). These females were nursing their pups for 3 days before litter removal. Besides, LIF expression levels were also determined using RNA from 3 days involuting glands from females that were inoculated with either saline solution (lane 6) or hydrocortisone (Hyd) (lane: 7) daily after litter removal. Lane 1 shows the standard LIF expression level from 33 days old virgin mouse mammary glands. Upper panel: Semi-quantitative comparison of LIF expression levels. Results are presented as a ratio of that detected in total mammary RNA from 33 days old virgins (fixed value=1). Each column represents the mean, and bars represent the SE, determined from 4 separate RT-PCR experiments. * indicates a significant increase in the relative LIF expression level in the mammary glands of surgically removed and sealed nipple compared with the expression level found in the contralateral intact ones ($P<0.001$). Lower panel: ethidium bromide stained gel showing one of the RT-PCR assays described above. B) LIF expression Northern blot analysis of #4 mammary gland poly-A RNA from: late pregnant mice (lane: 1), lactating intact glands (lane 2), intact glands 3 days after weaning (lane 3) and lactating glands in which their #4 teats have been sealed during 3 days (lane: 4). The blot was first hybridized with a LIF cDNA specific probe (upper panel), then stripped and hybridized with a GPDH cDNA specific probe (lower panel).

LIF induces cell death in the mammary gland epithelium and Stat3 activation

In order to study LIF protein effect on the mammary gland, LIF containing pellets were implanted in the lactating glands when endogenous expression is at its lowest level. Our goal was to determine whether the presence of exogenous LIF during lactation could induce events associated with involution while the mouse was still nursing its pups. Analysis of H&E stained slides from LIF pellet implanted glands showed no relevant morphological alterations. However, it has been demonstrated that the first stage of involution is characterized by apoptosis of the secretory cells rather than gland architectural alterations. Therefore, quantification of apoptosis was assessed morphologically in these sections (Fig.14 C, and inserts). Significantly more apoptotic nuclei were found in the LIF pellet implanted mammary glands: 3.92% ± 0.26% compared with control glands implanted with placebo pellets: 0.98% ± 0.22% (n=5, mean ± S.E. p=0.0019, Student's t-test for paired samples). Induction of mammary epithelium apoptosis by LIF pellet implantation was confirmed by TUNEL analysis (Figure 14): 3.62% ± 0.50% vs. 1.16% ± 0.5% (n=5, mean ± S.E. p=0.0017, Student's t-test for paired samples). color prints in the appendix

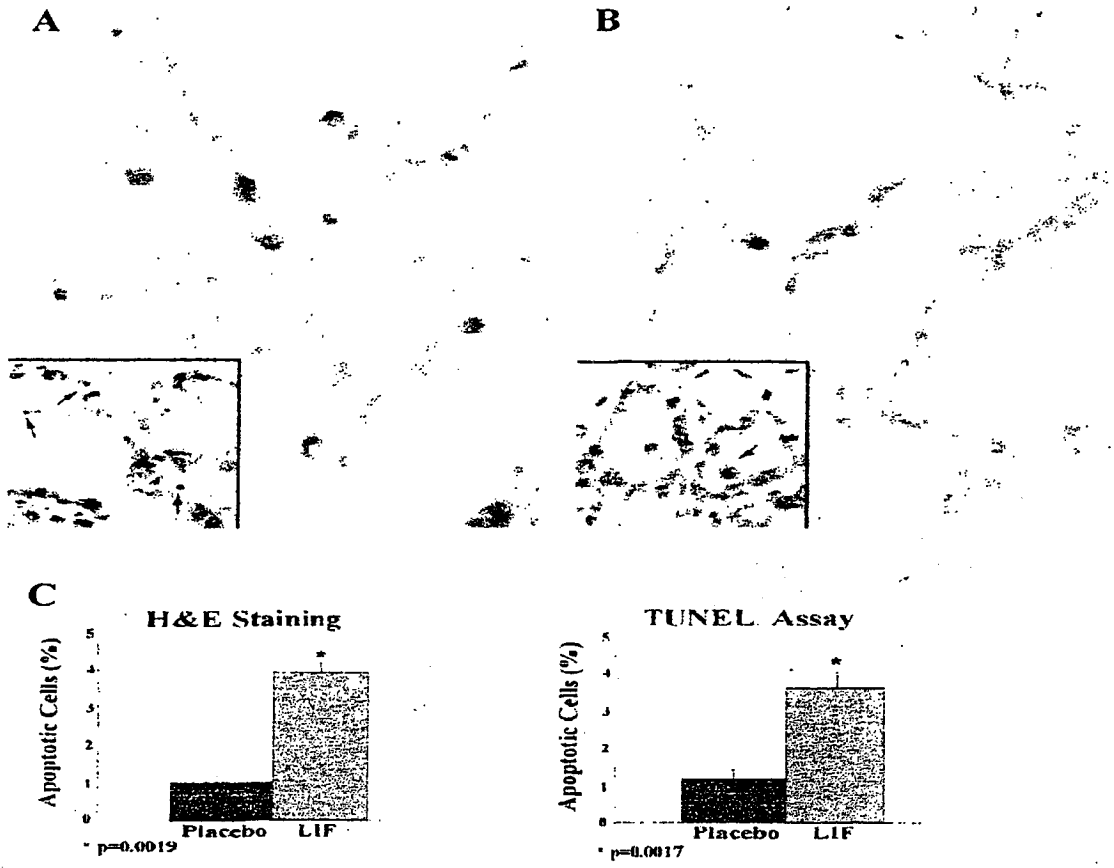

Figure 14: LIF induces apoptosis in lactating mammary glands

TUNEL staining of lactating mammary glands treated with LIF (A) or with placebo (B) pellets. TUNEL-positive apoptotic cells are identified as brown stained nuclei and they clearly predominate in A (magnification 400x). Arrows in the inserts show apoptotic cells in the same sections stained with H&E. Observations made by this method correlated with the results obtained by TUNEL assay. Quantification of the results (C) Each bar represents the mean of data collected from five independent samples. Error bars represent standard error of the mean.,Mean and SE were compared by Student's t-test for paired samples

It has been previously reported that LIF can activate Stat3 in several cell types and that phosphorylation of Stat3 (p-Stat3) is enhanced during the first three days of mammary gland involution. In contrast, very low p-Stat3 was found in pregnant and lactating glands (Chapman et al., 1999). Therefore, LIF ability to activate Stat3 transcription factor in the mammary gland was assayed in the LIF implanted pellet lactating glands. Phosphorylated Stat3 level was evaluated by immunoblot assays after Stat3 immunoprecipitation. LIF pellet implanted mammary glands showed a significant increase in Stat3 activation when compared with placebo pellet implanted glands: 62.2% ± 4.8% vs. 21.9% ± 8.1% relative to the highest level of Stat3 activation observed in 48 hour-involuting glands (n=4 mean ± S.E., p=0.005, Student's t-test). Lactating non-treated mammary glands were tested as negative controls (Fig. 15).

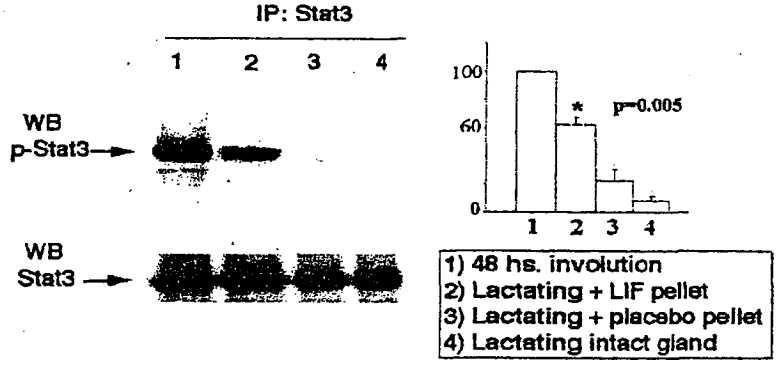

Figure 15: Effect of LIF on tyrosine phosphorylation of Stat3.
Immunoprecipitation and Western blot analysis of protein extracted from: intact 48 hours involuted mammary glands (lane 1), lactating mammary glands containing a LIF pellet (lane 2) or a placebo pellet (lane 3) and intact lactating glands (lane 4). Samples were immunoprecipitated with Stat3 antibody and immunoblotted with anti-phosphorylated Stat3 (p-Stat3) (Upper panel). Membranes were then stripped and reprobed with anti-Stat3 antibody (Lower panel). Graph: Quantification of the results of four independent mice. Each bar represents the mean and standard error calculated as a percentage of the value obtained in involuted gland in each blot. Mean and SE were compared by Student's t-test.

D. RESEARCH DESIGN AND METHODS:

Aim #1 **Progression of pregnancy-dependent tumors after dormancy in virgin females.**

We believe that the specific features that pregnancy-dependent tumors depict when they resume growth show clues that could lead us to the specific mechanisms involved in the surviving and fast progression of hormone-dependent mammary tumors after long-term dormancy: In our model as well as in other MMTV-induced, pregnancy-dependent tumor models (Sluyser & Van Nie, 1974), ER and PR expression are required for pregnancy-dependent tumor growth. However, our preliminar results show that ER and PR content are much lower in tumors that resumed growth after dormancy even though they required hormone stimulation to develop. Therefore, our hypothesis is that a small ER and PR positive population present in the dormant tumor provides specific signals (e. g. growth factors) that allow ER-PR- cells to rapidly grow and surpass the ER+PR+ slower cell populations. In order to test this hypothesis, $1mm^3$ fragments of HD tumors will be implanted in the mammary fat pad and will remain there for about 5 months in virgin hosts. These females will be then either impregnated or treated with estrogen and progesterone in order to allow tumor development. Right before impregnation and at different times during tumor development (for example once a week during the first four weeks of tumor development), tumor samples will be analyzed to identify the presence of ER+ and PR+ cells by immunohiostochemistry to identify their relative quantity and distribution during the dormancy period.

In these animals, at different times after implantation, Br dU uptake studies will be performed in order to determine whether the dormant tumors remain in G_0 or keep a low but detectable level of DNA synthesis (Wijsman et al., 1991).

It has been reported that breast invasive ductal carcinomas that show fibrotic foci have more aggressive characteristics than those without them (Hasebe, et al., 2001). Taking into account the high level of fibrosis observed in our model in tumors resuming growth after dormancy, proliferative activity of these active growing tumors will be analyzed by BrdU uptake as well as by PCNA and Ki-67 immunostaining. We will be then able to determine whether tumors that resumed growth after dormancy divide faster than the regular mammary tumor cells. In addition, double labeling (with hormone and growth factor receptors) will allow us to identify which factors would be involved in stimulating growth of these cell populations.

The presence of a rich stroma in tumors coming from dormancy periods suggests that factors belonging to the FGF (Fibroblast Growth Factor) family could be involved in dormant mammary tumor progression. We have chosen to analyze in our model 3 members from this family of proteins. The following paragraphs indicate the reason why these proteins were selected:

A) **bFGF /FGF2** In a new experimental model, it has been shown that fibrosis is associated with aggressive inflammatory breast cancer (Shirakawa et al., 2001). In this case, over-expression of bFGF/FGF2 has been observed. It has been also shown that production of this factor by pancreatic cancer cells induces ductal and stromal hyperplasia of the pancreas (Kuniyasu et al., 2001). Besides, bFGF/FGF3 has an important proliferative activity in different hormone—responsive mouse mammary tumor models (Jiang et al., 1993, Lamb et al., 1999).

B) **INT2/FGF3**. More than 15 years ago (Peters et al., 1983) it has been established that more than 50% of the mammary tumors arising in BR6 mice contain an acquired MMTV provirus integrated within a defined 25-kb domain of DNA on mouse chromosome 7 (Dickson et al., 1984). It was then found that the protein encoded by int-2 gene belonged to the Fgf family (Fgf3) (Smith et al., 1988). Mammary glands of virgin Fgf3 transgenic females appeared normal with only microscopic

areas of ductal hyperplasia composed of focal aggregates of cells. After parturition, the hyperplastic areas either regressed or remained static, becoming more pronounced in subsequent pregnancies. In this regard lesions resembled pregnancy-dependent lesions observed in BR6 mice and in our own model (Muller et al., 1990; Stamp et al., 1992). In addition, we have observed alteration of the *int2* locus in the 3 tumor lines tested (see "Preliminary results"); two of which have resumed growth after dormancy (T2312 and 2284). In the third one, the Fgf3/*int*2 insertion appeared early in the D2HD tumor (see Preliminary results). Then, it was not associated with HI pattern of growth and high fibrosis observed in the other two cases. However, it has to be noticed that this mutation is also present in the tumor belonging to this line that have resumed growth after dormancy. Then, over-expression of Fgf3/int2 could be necessary but not sufficient for displaying the phenotype observed in these tumors.

C) **FGF4.** It has been reported that Fgf4 transfected MCF-7 cells show a very important mitogenic activity that mask, if existant, their responsiveness to estradiol. In addition this factor induces secretion of VFGF that would be probably associated with the more aggressive phenotype observed in the MCF-7-Fgf4 cells. (Hajitou et al., 2000).

The expression of these genes will be analyzed in tumors that resumed growth after dormancy as well as their passages compared with HD and HI tumors that did not go through that process. The analysis will be made at the RNA level (Northern blot analysis) and protein level (Western blot analysis and Immunohistochemistry). Similarly, expression of FGF-R will be analyzed in these samples. In addition, *in situ* hybridization analysis will be carried out in dormant tumor samples right before and after hormone stimulation in order to identify the cell populations responsible for their production.

Another common trait that tumors that resumed growth after dormancy showed quite frequently is the presence of squamous metaplasia foci. This would indicate that in these tumors there are cells that suffer transdifferenciation into epidermis. In order to verify these events, the presence of keratins K1 and K5 will be investigated. In wild type epidermis, K1 is found in the spinous layer and K5 is observed preferentially in the basal layer and outer root sheath of hair follicles. K1 is not found in mammary epithelium and K5 is found in the myoepithelium surrounding ducts and alveoli. However, K1 and K5 are detected in hyperplastic structures and squamous cells upon activation of b-catenin (Miyoshi et al., 2002). Their expression patterns resembled those seen during epidermal differentiation. K1 and K5 immunostaining will be performed in pregnancy –dependent tumors, regular HI tumors and HI tumors that resumed growth after hormone stimulus. This will confirm that association between dormancy and transdifferentiation. In addition, since activation of b-catenin has been shown to induce transdifferentiation in mammary secretory cells, its level of expression and localization will be also tested in these tumors. In addition, as this protein is part of the *wnt* pathway, different steps of this cascade will be analyzed. Similarly, interaction with the lymphoid enhancer transcription factors (LEFs) and T cell factors (Tegs) followed by translocation of the entire complex to the nucleus will be analyzed. Expression and localization of the proteins involved will be studied by Western blot analysis, immunohistocehmistry, and microscopic visualization. Activation of the transcription factors will be analyzed by Electrophoretic Mobility Shift Assays *(EMSA)*.

In another hormone-dependent mouse mammary tumor model, it has been shown that administration of phosphothiolated antisense oligonucleotides against the IGF-I receptor efficiently inhibit tumor growth (Salatino et al., 2001). In our model, we will determine whether blocking FGF-R by this approach would affect tumor growth stimulation after dormancy. These authors report that the most efficient treatment is to inoculate the oligonuclotides in the tumor body (100mg/day per 12 days). In our case, both #4 fat pads will be implanted with tumor fragments, after 5 months, the female mouse will be impregnated. When pregnancy is detected, one of the #4 mammary fat pads will be treated with

the antisense oligonucleotides, while the other one will be treated with placebo. Then, tumor growth in both flanks will be compared. Histological features as well as proliferative activity (by mitosis count, PCNA and/or Ki 67 staining), apoptosis (by morphological as well as TUNEL analysis) will be evaluated in these tumors.

Problems and alternative experiments: We do not expect major problems with the first phase of studies proposed in this aim. First, we have to characterize tumors that went through a period of dormancy and their successive progression. Therefore, the main problem could be technical as the possibility of the target proteins to be identified by the antibodies used, i.e. antibodies quality, antigen stability, fixation of the tissue samples, etc. A more difficult problem to solve would be blocking the action of the investigated growth factors *in vivo* in order to test their activity. Inoculation of antisense sequences has been successful in other tumor models, however we will have to set up the conditions for our experiments, being a new approach this could be the major source of trouble. Another approach to evaluate the activity of the growth factors involved can be done by using KO animals (or conditional KO animals) when available. In that case we could also determine whether these growth factors are provided by the tumor tissue or by the host.

Aim #2: **Cloning and sequencing MMTV insertion site in order to find new genes associated to tumor progression**

Our goal is to continue the isolation and cloning of MMTV(LA) insertion sites following the procedure described in **"Preliminary Results"**. We are specially interested in cloning out insertion sites associated to acquisition of hormone-independency in order to identify genes involved in that process.

Besides the obvious opportunity of finding new genes associated with mammary tumorigenesis and/or tumor progression, cloning and sequencing regions next to insertions found in HI tumors will make possible to investigate by PCR analysis whether this insertion was already present in the HD original tumor although not detectable by Southern blot analysis. This will allow us to learn whether the insertion sites observed in HI tumors are already present in a small underrepresented subpopulation of the HD tumor that are selected during tumor progression or they are newly acquired one that induce a fast change in tumor development.

Upon finding MMTV affected loci, expression of putative coding regions near the insertion sites will be evaluated by Northern blot analysis. In addition, using specific probes for the mouse genomic region next to the insertion site we will determine whether that area is commonly affected by MMTV insertions in our tumor pool by Southern blot analysis. In case we confirm that a putative gene expression is affected by the insertion, the other tumors will be also tested for altered expression by Northern blot analysis and/or RT-PCR. In this case also, normal epithelial cell lines (like NMuMG or HC11 cells) will be transfected with expression vectors containing the either altered or over-expressed sequences, in order to determine whether any change in cellular behavior is observed.

This part of my project is the one we believe can provide a very rich interaction with Drs. Gilbert H. Smith and Robert Callahan. First, we can interchange data about MMTV altered loci in both mouse colonies in order to amplify the screening for different MMTV strains and mouse genetic background. Besides, if we find an interesting new altered locus in our model in Argentina, a transgenic mouse could be developed in collaboration with their groups. Finally, Dr. Callahan has suggested the possibility to compare the sequences of the different MMTV strains carry the mice in both colonies and

those already known by the literature, in order to find out a pattern that could associate the ability of different mouse strains to induce either pregnancy-dependent or independent mammary tumors.

Problems and alternative experiments: The first problem you face trying to clone out fragments obtained from I-PCR reactions is to decide whether or not the fragments obtained correspond to what we see in the Southern blot analysis. We know the PCR amplified fragment has to be the same size as the one the detected by Southern blot analysis minus the distance between the primer used for amplification. However, as we work with fragments that range from 2 to 10kb (see figures above) it is not easy to have a very precise estimation of PCR fragment size from its visualization in the BrEt stained gels. For example, in tumor D2 HI (see above), cloning out the 2 kb fragment that we found corresponded to a single MMTV-LTR sequence inserted in the *int2/Fgf* area was relatively easy. However, when we tried to identify the 4kb (aprox.) associated with progression with hormone-independency we found that we re-cloned the 2kb fragment ligated with something else during the circularization of DNA. Therefore, the only way to reduce the possibility of ligation of different fragment to occur is to dilute the restricted DNA solution as much as possible. In some occasions the DNA solution that is used as DNA template for PCR is so diluted that we need a nested PCR to be able to see in a BrEt stained gel the reaction product. To this second PCR round we would use downstream primers that would hybridize with MMTV sequences, but that do not need to be specific for MMTV (LA) since specificity is provided by the PCR first round. Nevertheless, in the case more than one Eco RI fragment get trapped in the circle and amplified by PCR, this can be detected by Eco RI digestion of I-PCR products, before or after being ligated to the cloning vector. Another problem we will face, trying to clone out bigger fragments (6-12 kb) is the fact that these fragments do not get easily trapped by the pGemT vector we are using. In that case we have to take into account that the necessity of using MMTV (LA) specific primers plays against the necessity of making the cloning fragment as small as possible. Therefore, once we have obtain the specific PCR fragment, we can re-amplify these sequences using primers next to the end of the MMTV-LTR and to the EcoRI site in the virus sequence. This way, we will be able to get a significant lower size fragment. Another technical approach to this problem is to design MMTV (LA) specific primers with restriction endonuclase sequences at the 5'end of them. Then we will have the possibility to ligate them to other cloning vectors that accept bigger fragments than pGemT does. Another way to solve this problem could be to re-design the I-PCR reaction using a four base cutter instead of a six base restriction enzyme, in order to get smaller fragments to clone out. This approach would require new Southern blot analysis to determine the fragment size of our new cloning targets.

Aim #3: LIF and involution:

Since we have demonstrated a tight relationship between LIF expression and the first phase of mammary involution we would like now to get a deeper knowledge of the mechanisms involved in these events. Therefore, we will check the level of expression of genes associated with apoptosis (either induction or protection) that could interact during transition from lactation to involution (Metcalfe et al., 1999; Lund et al., 1996). Then, expression levels of a number genes like Bax, Bad, Bcl-2, Bcl-x, ICE will be tested in lactating mammary glands implanted with LIF pellets as well as in mammary epithelial cell lines as HC11 and NMuMG treated with this factor. On the other hand, it has been demonstrated that NFkB has the same temporal pattern of activation as the one we have observed for LIF expression during mammary involution (Clarkson et al., 2000). Therefore, in order to determine whether LIF treatment of the mammary gland could induce NFkB activation, immunostaining of mammary tissue preparations originated in mice treated or not with LIF implants will be performed. Antibodies against specific members of the kB family will be used (Clarkson et al., 2000).

A possible stimulus for LIF induction in the mammary gland during involution could be associated with mammary cells stretching due to milk retention when pups stop suckling. As a matter of fact, stretching of cardiac myocytes induce Stat 3 activation as well as LIF (Pan et al, 1999). A more recent report indicates that LIF activity on these cells would be mediated through the MAPK pathway (Nicol et al, 2001). Therefore, we will determine whether milk retention in the lobular alveoli, due to lack of pups nursing, induce activation of MAPK cascades in normal mammary tissue, as reported before for cardiomyocites. On the other hand, LIF-induced apoptosis has been associated with the activation of the c-Jun Kinase (JNK, a MAPK sub-family member) signaling pathway (Savitz and Kessler, 2000). Therefore, nipples of lactating mammary glands will be sealed with glue in order to interfere with milk efflux in those glands and activation of the MAPK and JNK pathways will be tested in these glands. Phosphorylation of proteins involved in signaling pathways will be determined by immunoprecipitation and Western blot analysis with antibodies that recognize the active, phosphorylated forms. Alternatively, specific kinase assays can be performed to assess MAPK and JNK activity (Coso et al., 1995)

In order to obtain a better understanding of LIF activity on mammary epithelial cells, NMuMG and HC11 cell lines will be treated with LIF. Then, the proliferative activity as well as apoptotic induction will be tested. The first one will be determined by Timidine-^{3}H uptake. Apoptosis will be evaluated by TUNEL assay, DNA ladder, BrI, Acridine Orange and/or Annexin V assay. In the LIF treated cell lines, the Stat 3 activation as well as the kinase cascades that were activated by LIF pellet in vivo will be tested to determine whether their activation is due to a direct activity of LIF on the epithelial cells.

In order to study the impact that cell stretching can exert on mammary epithelium, mammary primary cultures as well as cultured mammary cell lines like NMuMG and HC11 will be stretched as previously described for cardiomyocytes and fibroblasts (Lee et al., 2000; Komuro et al., 1996). During that stretching we will analyze LIF expression, NFkB activity and induction of the MAPK -JNK signaling pathways.

In case we determine that a kinase pathway is activated by sealing the nipple and possibly by stretching of the epithelial cell lines, but no activation is obtained by either LIF treatment of these cultured cells nor by LIF *in vivo* treatment of the mammary gland, we can hypothesize that these pathways can be inducing LIF expression instead of being the target of downstream signaling resulting from the association of LIF with its receptor. In order to test this last hypothesis, HC11 and/or NMuMg cells will be co-transfected with an expression vector containing a reporter gene (β-galactosidase/luciferase/CAT) driven by the mouse LIF promoter (Gollner et. al., 1998), and vectors that express MAPK or JNK activating molecules. Over-expression of the reporter gene resulting from activation of MAPK/JNK will be indicative of MAPK/JNK as mediators of signaling to the LIF promoter.

In order to test whether the LIF-activated pathways are altered in mammary tumor cells, studies will be carried out using the MMTV-induced mouse mammary tumors as targets for LIF activity. *In vivo*, the tests will be carried out in tumor implants that will develop in mammary fat pads implanted with LIF-pellets. *In culture,* similar experiments to those that were described in NMuMG or HC11 cells will be carried out in mammary tumor primary cultures. Then, it will be analyzed whether LIF treatment induces Stat3 activation, apoptosis and the different pathways tested in normal mammary epithelia (see above). The effect of blocking LIF expression in mammary tumor cells will be tested by treatment with LIF antisense oligo-nucleotides inoculated in the mammary fat pad where the tumor implant has been implanted.

Problems and alternative experiments:

The experiments proposed in this section are quite simple and straightforward. However, we are aware that because of their novelty, at least for the background and experience of our group, they will require to be set up. We are not sure about the efficiency of measuring the MAPK and JUNK cascades in the mammary gland *in vivo*. In addition, we will have to set up the conditions for measuring the effect of stretching on mammary epithelial cells, we have crafted a silicon culture plate of variable lengths that is currently under testing. The other experiments, although they involve establishing new techniques in our laboratory, have been widely used in many other models so I do not have any doubts that we will be able to determine the right conditions to pursue this line of work.

g. Human Subjects Research

NA

f. Vertebrate animals

1. In this project, BALB/c female mice will be used. Number and justification of its use will be specified for each aim:

Aim #1 Progression of pregnancy-dependent tumors after dormancy in virgin females

Most of the labeling for specific keratins and FGF's of tumors that developed after long term dormancy will be performed in already collected material so no more mice will be used for the characterization of these tumors.

In order to follow tumor dormancy and development right after impregnation, 6 female mice will be used to implant small pieces of pregnancy-dependent tumors in their #4 mammary gland fat pads. Tumor tissue to be implanted has been already obtained and frozen in liquid nitrogen. Four females will be impregnated 5 month after tumor implantation (2 will be left as control to confirm there is no tumor development without hormone-stimulation). BrdU uptake, morphological and immunostaining of tumor samples will be performed in the developing implanted tumors. We believe this experiment should be repeated at least 3 times, using therefore about 18 mice with this purpose. Another 2/3 sets of experiments should be performed when FGF-R antisense oligonucleotide activity on tumor growth is tested. This will take then another 12/18 animals.

Aim #2: Cloning and sequencing MMTV insertion site in order to find new genes associated to tumor progression.

For pursuing this goal, we will use mostly frozen material from tumor tissue we have already obtained from the 17 *in vivo* lines we have developed. As described in "**Preliminary results**", most of these lines comprehend pregnancy-dependent as independent variants, and six of them also have variants that correspond to tumor development after dormancy periods. We believe we have enough frozen material to do all the DNA and also RNA and protein work that involve tumor cloning as well as testing the expression of the affected genes. However, in case we would need more material, frozen tissue kept in medium with glycerol in liquid nitrogen will be subcutaneously implanted in BALB/c female mice in order to recover tumor tissue.

Aim #3: Mammary apoptotic signals during early involution.

In our previous experiments, for investigating LIF effect on mammary lactating tissue. LIF containing pellets had been implanted in the left #4 mammary glands of nursing females. On the right #4 mammary gland, placebo-containing pellets were implanted. For positive control, involuting glands were used, while for negative control intact glands from nursing females were utilized. Apoptosis level

had been determined in formalin fixed and paraffin embedded tissue from these glands. For the project presented herein, all the proposed immunohistochemical analysis can be carried out in new slides from those paraffin blocks so we won't need to sacrifice more animals for those assays.

For all the other testing involving RT-PCR, Northern blot and Western blot analysis in LIF pellet and placebo implanted glands, we consider that another 10 BALB/c female mice will be necessary.

In addition LIF effect will be also evaluated on mammary growing tumors in LIF and placebo pellet implanted fat-pads. We believe that each of these assays could involve around 5 animals. Depending on the results, we will test more than a tumor line. Then, number of total animals could rise to 15 for these experiments.

For testing the activation of different signaling pathways when mammary glands are sealed during lactation, we estimate that another 6 to10 BALB/c female mice would have to be used.

All the other experiments proposed in this section involve cell lines and/or primary mammary cultures. In case we find out that primary cultures will be necessary, more BALB/c mice will be required. These numbers are very difficult to establish because we have not started yet the setting up of these assays. However, I believe that a serious first approach could take around 10 mice.

In summary, I believe that during the whole project, 75 to 100 mice (taking into account illnesses, unexpected deaths, etc.) will be utilized.

2. BALB/c mice will be used because this is the mouse strain in which the MMTV (mouse mammary tumor virus) variants used in this project were discovered and their tumorigenic activity described. It is widely known the importance of using *in vivo* models to study tumor progression. For example, we have found several interesting histological features associated with tumor growth resumption that could be revealing tumor progression mechanisms that cannot be simulated *in culture*. Similarly, there is no *in vitro* model that faithfully reproduces mammary gland involution. Nevertheless, those aspects that can be studied at the cellular or molecular in *in culture* or *in vitro* models, will be carried out in such a way. We believe that the paragraph above shows a fair calculation of the expected number of mice to be used in the proposed experiments. We do not believe that this is an excessive number and our mouse colony can supply such an amount.

Balb/c mice from our mouse colony are maintained in a pathogen-free temperature-controlled environment. They are housed 4 per cage in air conditioned rooms a t 20 $\pm$ 2^{0}C, keep under an automatic 12h light/12 hour darkness schedule and given sterilized laboratory chow and water *ad libitum* in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

3. The animals are under permanent professional veterinarian care. Dr. Héctor Costa (MV) is our full time professional assistant, member of the CONICET (National Council of Scientific and Technological Research) Assistant Career, who takes care of the animal health. Besides, there are another 5 non-professional employees who take care of the different issues of our animal facility.

Our animal facility has been already approved by NIH. A Fogarty International Research Collaborative Award (FIRCA #5-34881; PIs: Isabel Piazzon Ph. D in Argentina, Susan Ross, Ph. D. at the University of Pennsylvania in USA) has been awarded two years ago for experimental work that also involves our mouse colony.

4/5 All the surgical procedures, including pellet and tissue implantation, will be performed previous inoculation of Nembutal as anesthetic. During surgery, the animals will be movement restrained without hurting their limbs. When necessary, euthanasia will be performed by cervical dislocation or CO_2 inhalation.

g. Literature cited.

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h. Consortioum/ Contractual Arrangments:

The project presented herein will be carried out at the Division of Experimantal Medicine, Institute of Hemathological Research (IIHEMA), National Academy of Medicine of Buenos Aires, Argentina (División de Medicina Experimental, Instituto de Investigaciones Hematológicas, Academia Nacional de Medicina de Buenos Aires). The programmatic and administrative personnel of this non-profit organization are aware of the NIH consortium agreement policy and are prepared to establish the necessary inter-organizational agreements consistent with that policy.

i. Consultant and Reference letters

Herein I present the faxed copies of the reference letters from my former supervisors, Drs. Gilbert H. Smith and Robert Callahan, as required specifically for the "Global Health Research Initiative Program for New Foreign Investigators" (the original letters are being sent directly by Drs. Smith and Callahan). They also agree in becoming consultants and collaborators in the project described above. The third required letter of reference is provided by Dr Christiane Dosne Pasqualini, Scientific Director of our Institute (IIHEMA). In addition, a fourth letter from Dr. Omar Coso is included to show his role as consultant for our project.

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April 16, 2002

To Whom It May Concern

Dr. [redacted] was a Research Fellow in The Oncogenetics Section for 5.5 years (March,1992-September, 1997). In this position she worked with Dr. Gilbert H. Smith and myself. During this period she became a highly skilled molecular geneticist, who demonstrated an ability to perform research at an outstanding level in the area of mammary gland development and tumorigenesis. Dr. [redacted] was a highly productive scientist. The results of her research has been described in eight publications, four of which she was the first author. Furthermore, she was asked to present her work at seven international meetings during her stay here. Since returning to Argentina she continues to be highly productive. Dr. [redacted] and her colleagues have developed a novel mouse strain that develops mouse mammary tumor virus (MMTV) induced pregnancy dependent mammary tumors. She proposes to use the MMTV genome as a molecular tag to identify flanking genes whose expression has been altered as a consequence of viral integration. Dr. Smith and I anticipate collaborating with Dr. [redacted] on this project at the level of nucleotide sequence analysis and chromosomal localization integrated MMTV genomes

Sincerely yours,

Robert Callahan, Ph.D.
Chief, Oncogenetics Section
Basic Research Laboratory
National Cancer Institute

DEPARTMENT OF HEALTH & HUMAN SERVICES Public Health Service

National Institutes of Health
National Cancer Institute
Bethesda, Maryland 20892

April 16, 2002

To whom it may concern,

Dr. [redacted] was a Fogarty fellow in my laboratory from The Spring of 1992 to the Autumn of 1997. During her tenure she was very active in the laboratory, producing 4 first author papers and co-authoring 4 others. Since her return to Buenos Aires, she has continued to be an innovative and productive scientist in her own laboratory, where she supervises several junior scientists. She has developed a novel mammary tumorigenesis model wherein tumors arise during pregnancy in the mammary gland but resorb during lactation. In a recent paper, she and her co-workers demonstrate that these tumors contain numerous estrogen receptor and progesterone receptor-positive cells. This is unusual in mouse mammary cancer models and represents a unique opportunity to evaluate the role of estrogen and progesterone in a tumor progression model in mice. In addition, she has shown that these hormone-dependent mammary tumors begin as polyclonal cellular population, which subsequently become monoclonal during progression to a hormone-independent state. Her laboratory is also engaged in discovering the genetic mutations that are associated with this model. Using the mouse mammary tumor virus (MMTV) as a molecular tag, she plans to isolate sequences flanking MMTV-induced insertional mutations within the hormone-dependent tumors and to sequence these regions with the aim of identifying the affected gene. It is our plan to collaborate with her in this venture by providing support for the sequencing and chromosomal localization of the MMTV-induced mutations.

Sincerely Yours,

Gilbert H. Smith, Ph.D., Chief
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TO WHOM IT MAY CONCERN [redacted]

It is a pleasure for me to recommend Dr. [redacted] whom I have known for over 14 years. She came to this Institute as a young student and did her research work on progesterone-induced mammary tumors with Dr. Claudia Lanari who directed her Ph.D. thesis in 1992. Her achievements during those years yielded several publications. She then spent several years as a post-doc at the National Cancer Institute where she did very good work. She always remained in close contact with this Institute to which she eventually returned a few years ago. She had become a fully developed scientist who is now leading a research group which is producing valuable data continuing with her interest in mammary gland development and involution.

[redacted] has proved to be a performer above average in every step of her career. She graduated with high scores and immediately fitted in when she started during research at the bench, earning the respect of colleagues and students. Her commitment to continuous progress during this difficult economic crisis has led her to apply for research grants from foreign prestigious institutions.

For all these reasons I fully support her application with the conviction that being an NIH grantee would be an award that Edith fully deserves.

Please feel free to contact me should you need more information.

Ch Pasqualini

Christiane Dosne Pasqualini, Ph.D.
Scientific Director

e-mail: [redacted]

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Buenos Aires, April 10th, 2002

To whom it may concern!

It is with great pleasure that I write this letter to support the application presented by Dr. [redacted].

I certify that due to our ongoing collaborative projects we will provide Dr. [redacted]'s group with DNA reagents and antibodies in order to perform MAPK assays. Our expertise in the field combined with their deep knowledge of the mammary glands will allow us together to dissect the signaling pathways that link LIF, the MAPKs and transcription factors during the apoptotic process.

The necessary equipment necessary to get the results described in her proposal is available at both her institution and our own, so it is my opinion that there will be no problem in developing this project in which I act as a consultant.

Should you need any more information do not hesitate to contact me.

Sincerely.

Omar Adrian Coso Ph.D.

CHECKLIST

TYPE OF APPLICATION *(Check all that apply.)*

☒ NEW application. *(This application is being submitted to the PHS for the first time.)*

☐ SBIR Phase I ☐ SBIR Phase II: SBIR Phase I Grant No. _____ ☐ SBIR Fast Track

☐ STTR Phase I ☐ STTR Phase II: STTR Phase I Grant No. _____ ☐ STTR Fast Track

☐ REVISION of application number: _____

(This application replaces a prior unfunded version of a new, competing continuation, or supplemental application.)

☐ COMPETING CONTINUATION of grant number. _____

(This application is to extend a funded grant beyond its current project period.)

INVENTIONS AND PATENTS *(Competing continuation appl. and Phase II only)*

☒ No ☐ Previously reported

☐ SUPPLEMENT to grant number: _____

(This application is for additional funds to supplement a currently funded grant.)

☐ Yes. If "Yes," ____ ☐ Not previously reported

☐ CHANGE of principal investigator/program director.

Name of former principal investigator/program director: _____

☒ FOREIGN application or significant foreign component

1. PROGRAM INCOME ***(See instructions.)***

All applications must indicate whether program income is anticipated during the period(s) for which grant support is request. If program income is anticipated, use the format below to reflect the amount and source(s).

Budget Period	Anticipated Amount	Source(s)

2. ASSURANCES/CERTIFICATIONS ***(See instructions.)***

The following assurances/certifications are made and verified by the signature of the Official Signing for Applicant Organization on the Face Page of the application. *Descriptions of individual assurances/* certifications are provided in Section III. If unable to certify compliance, where applicable, provide an explanation and place it after this page.

•Human Subjects; •Research Using Human Embryonic Stem Cells• •Research on Transplantation of Human Fetal Tissue •Women and Minority Inclusion Policy •Inclusion of Children Policy• Vertebrate Animals• •Debarment and Suspension; •Drug- Free Workplace *(applicable to new [Type 1] or revised [Type 1] applications only)*; •Lobbying; •Non-Delinquency on Federal Debt; •Research Misconduct; •Civil Rights (Form HHS 441 or HHS 690); •Handicapped Individuals (Form HHS 641 or HHS 690); •Sex Discrimination (Form HHS 639-A or HHS 690); •Age Discrimination (Form HHS 680 or HHS 690); •Recombinant DNA and Human Gene Transfer Research; •Financial Conflict of Interest (except Phase I SBIR/STTR) •STTR ONLY: Certification of Research Institution Participation.

3. FACILITIES AND ADMINSTRATIVE COSTS (F&A)/ INDIRECT COSTS. See specific instructions.

☐ DHHS Agreement dated: _____ ☒ No Facilities And Administrative Costs Requested.

☐ DHHS Agreement being negotiated with _____ Regional Office.

☐ No DHHS Agreement, but rate established with _____ Date _____

CALCULATION* *(The entire grant application, including the Checklist, will be reproduced and provided to peer reviewers as confidential information.)*

a. Initial budget period:	Amount of base $	_____	x Rate applied	_____	% = F&A costs	$	_____
b. 02 year	Amount of base $	_____	x Rate applied	_____	% = F&A costs	$	_____
c. 03 year	Amount of base $	_____	x Rate applied	_____	% = F&A costs	$	_____
d 04 year	Amount of base $	_____	x Rate applied	_____	% = F&A costs	$	_____
e. 05 year	Amount of base $	_____	x Rate applied	_____	% = F&A costs	$	
					TOTAL F&A Costs	$	

*Check appropriate box(es):

☐ Salary and wages base ☐ Modified total direct cost base ☐ Other base *(Explain)*

☐ Off-site, other special rate, or more than one rate involved *(Explain)*

Explanation *(Attach separate sheet, if necessary.)*:

4. SMOKE-FREE WORKPLACE ☒ Yes ☐ No *(The response to this question has no impact on the review or funding of this application.)*